DESIGN, FABRICATION AND CHARACTERIZATION OF THROMBOLYTIC ACTIVITY OF BAUHINIA RACEMOSA EXTRACT LOADED NANOEMULSION.

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

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In partial fulfillment of the requirements for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICS

Submitted by

SHRUTI RAMESH TIMANE

Reg no: 261410763

Under the guidance of

Mr. AKELESH.T, M. Pharm, DIH
Assistant Professor
Department of Pharmaceutics
R.V.S College of Pharmaceutical Sciences
Sulur, Coimbatore

RVS COLLEGE OF PHARMACEUTICAL SCIENCE

SULUR, COIMBATORE,TAMILNADU

OCTOBER 2016

Certificate

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Mr. AKELESH. T, M. Pharm, DIH
Assistant Professor
Department of Pharmaceutics
RVS College of Pharmaceutical Sciences
Sulur, Coimbatore-641402

Date:

Place: Coimbatore

Certificate

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Dr. R. Manavalan, **M. Pharm, Ph.D**
Professor and Head
Department of Pharmaceutics
RVS College of Pharmaceutical Sciences
Sulur, Coimbatore.

Date:

Place**: Coimbatore**

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Dr. R. Venkatanarayanan, M. Pharm, Ph.D

Principal

RVS College of Pharmaceutical Sciences

Sulur, Coimbatore-641402

Date :

Place: **Coimbatore**

Certificate

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Mr. AKELESH.T, M.Pharm, DIH.,

Assistant Professor

Department of Pharmaceutics

R.V.S College of Pharmaceutical Sciences

Sulur, Coimbatore

Dr. R. Manavalan

Professor and Head

Department of Pharmaceutics

R.V.S College of Pharmaceutical Sciences

Sulur, Coimbatore

Dr. R.VENKATANARAYANAN, M.Pharm., Ph.D.,

Principal

RVS College of Pharmaceutical Sciences,

Sulur, Coimbatore-641402

Evaluation Certificate

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Name of the Candidate	:	**SHRUTI RAMESH TIMANE (Reg No:261410763)**
Course of Study	:	**Master of Pharmacy in Pharmaceutics**
Institution Name	:	**RVS College of Pharmaceutical Sciences, Sulur, Coimbatore**

INTERNAL EXAMINER **EXTERNAL EXAMINER**

Declaration

I hereby declare with immense pleasure and satisfaction that this dissertation work entitled" **DESIGN, FABRICATION AND CHARACTERIZATION OF THROMBOLYTIC ACTIVITY OF *BAUHINIA RACEMOSA* EXTRACT LOADED NANOEMULSION"** was carried out by me under the guidance of **Mr. AKELESH.T,** M.Pharm, DIH, Assistant Professor, Department of Pharmaceutics, RVS College of Pharmaceutical Sciences, Sulur, Coimbatore.

SHRUTI RAMESH TIMANE

Reg.No: 261410763

II. M.Pharm

Department of Pharmaceutics

R.V.S College of Pharmaceutical Sciences.

Sulur, Coimbatore-641402.

Date:

Place: Coimbatore.

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Principal
RVS College of Pharmaceutical Sciences
Sulur, Coimbatore-641402

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Institution Name	:	**RVS College of Pharmaceutical Sciences, Sulur, Coimbatore**

INTERNAL EXAMINER **EXTERNAL EXAMINER**

Date:

Place:

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SHRUTI RAMESH TIMANE

LIST OF ABBREVIATIONS

1	%	Percentage
2	&	And
3	° C	degree Celsius
4	Mg	Microgram
5	Mm	Micrometer
6	o/w	Oil in water
7	w/o	Water in oil
8	Nm	Nanometer
9	Gm	Gram
10	SD	Standard deviation
11	DDS	Drug Delivery System
12	**F**	Formulation
13	Sec	Second
14	Hr	Hour
15	Mg	Milligram

16	Min	Minute (s)
17	Kg	Kilogram
18	UV	Ultraviolet Visible
19	w/v	Weight/volume
20	RH	Relative humidity
21	SEM	Scanning Electron Microscope
22	Ml	Millilitre
23	WHO	World Health Organisation
24	OECD	Organization Economic Cooperation and Development
25	CPSCEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
26	Hb	Haemoglobin
27	WBC	White Blood Corpuscle
28	RBC	Red Blood Corpuscle
29	SGPT	Serum Glutamic Pyruvic Transaminase
30	SGOT	Serum Glutamic Oxaloacetic Tranaminase

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ABSTRACT

The main objective of the study is to develop a design to formulate and characterize the nanoemulsion which is loaded with *Bauhinia racemosa* crude leaf extract. The leaves are found to possess thrombolytic activity.

The nanoemulsion was prepared by using Tween 80, ethanol, cinnamon oil, distilled water in varying ratios of 2:1, 3:1, 4:1. The prepared nanoemulsion was then evaluated for particle size distribution, zeta potential, particle morphology, release study and stability studies.

From these results it was concluded that the nanoemulsion containing particles was within the size range and also have a release profile that exhibit a uniform prolonged release pattern.

Key words: Bauhinia racemosa, Nanoemulsion, solubility.

भारत सरकार / GOVERNMENT OF INDIA
पर्यावरण एवं वन मंत्रालय / MINISTRY OF ENVIRONMENT & FORESTS
भारतीय वनस्पति सर्वेक्षण/ BOTANICAL SURVEY OF INDIA

दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
टी,एन.ए.यू कैम्पस / T.N.A.U. Campus
लाउली रोड / Lawley Road
कोयंबत्तूर/ Coimbatore - 641 003

टेलीफोन / Phone: 0422-2432788, 2432123, 2432487
टेलीफक्स/ Telefax: 0422- 2432835
ई-मैल /E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No. BSI/SRC/5/23/2016/Tech./585 **दिनांक**/Date: 22nd April 2016

सेवा में / To

Miss. Shruti Ramesh Timane
M. Pharmacy (Pharmaceutics)
RVS College of Pharmaceutical Sciences
Trichy Road, Sulur
Coimbatore - 641402

विषय/Sub.: Authentication of Plant Specimen – reg.

महोदया/Madam,

The plant specimen brought by you for identification is identified as *Bauhinia racemosa* Lam. – CAESALPINIACEAE). The specimen returned herewith for preservation in their college / Department / Institution Herbarium.

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

22/4/16

(**डा. एम. पलनिसामी** /Dr. M. Palanisamy)
वैज्ञानिक 'डी', प्रभारी /Scientist 'D', In-charge
वैज्ञानिक 'डी' / SCIENTIST 'D'
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
दक्षिणी क्षेत्रीय केन्द्र
Southern Regional Centre
कोयम्बत्तूर / Coimbatore - 641 003.

INTRODUCTION

1.1 Nanoemulsion. [1,2]

A controlled release drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic effect of a given drug. To obtain maximum therapeutic safety and efficacy, it becomes necessary to deliver the agent to the target tissue in the optimal amount in the right period of time there by causing little toxicity and minimal side effects. There are various approaches in delivering a therapeutic substance to the target site in a sustained controlled release fashion. One such approach is using nanoemulsion as carriers for drugs.

Nanoemulsions are submicron sized emulsions that are under extensive investigations as a drug carrier for improving the delivary of therapeutic agents. Nanoemulsions are by far most advanced nanoparticle system for the systemic delivary of biologically active agents for controlled drug delivary and targeting. Nanoemulsions are thermodynamicaly stable isotropic system in which two immiscible liquids (water and oil) are mixed to form a single phase by means of an appropriate surfactant or its mix with a droplet diameter of approximately 5nm - 200nm.Because of small size nanoemulsion are transparent.

1.2 Types of nanaoemulsion. [3]

There are three types of nanoemulsion which can be formed:

(a) oil in water nanoemulsion in which oil is dispersed in the continuous aqueous phase,

(b) water in oil nanoemulsion in which water droplets are dispersed in continuous oil phase,

(c) bi-continuous nanoemulsions where in micro domains of oil and water are interdispersed within the system.

The main difference between emulsion and nanoemulsion are that even though emulsion is having kinetic stability they are thermodynamically unstable. Emulsions are cloudy but nanoemulsions are clear and translucent. They also differ in their method of preparation.

1.3 Advantages of nanoemulsion.[1]

a. It may be used as substitute for liposomes and vesicles
b. It improves the bioavailability of drug
c. It is non-toxic and non-irritant in nature.
d. It has improved physical stability.
e. Nanoemulsions have small-sized droplets having greater surface area providing greater absorption.
f. It can be formulated in variety of formulations such as foams, creams, liquids, and sprays.
g. It provides better uptake of oil-soluble supplements in cell culture technology.
h. It helps to solubilize lipophilic drug.
i. Helpful in taste masking.
j. Less amount of energy is required.

1.4 Disadvantages of nanoemulsion. [4]

a. Use of a large amount of surfactant and co-surfactant necessary for stabilizing the nano droplet
b. Limited solubility capacity for high melting substance.
c. The surfactant must be non toxic for pharmaceutical application.
d. Nanoemulsion stability is influenced by environmental parameters such as temperature and pH.

1.5 Components of nanoemulsion.[1]

The main components of nanoemulsion are oil, emulsifying agents, and aqueous phases.

Oils can be of any type like castor oil, corn oil, coconut oil, evening primrose oil, linseed oil, mineral oil, olive oil, peanut oil, etc. A mixture of oil and water may yield a crude temporary emulsion, which upon standing, will separate in two distinct phases due to the coalescence of the dispersed globules.

Emulgents or emulsifying agents can impart stability to such systems. Emulgents are broadly classified as surfactants like spans and tweens, hydrophilic colloids such as acacia and finely

divided solids, e.g., bentonite and veegum. An emulgent, in addition to its emulsifying properties, should be nontoxic and its taste, odour and chemical stability should be compatible with the product.

Some of the desirable properties of an emulgent are:

(1) It should be able to reduce the surface tension to below 10 dynes/cm,

(2) It should be adsorbed rapidly around dispersed phase globule to form a complete and coherent film to prevent coalescence,

(3) It should help in building up an adequate zeta potential and viscosity in the system so as to impart optimum stability, and

(4) It should be effective in a fairly low concentration.

Emulgents form monomolecular, multimolecular or particulate films around the dispersed globules.

1.5.1 Monomolecular films

Surfactant type of emulgents stabilizes a nanoemulsion by forming a monolayer of adsorbed molecules or ions at the interface reducing interfacial tension. In modern day practice, combination of emulgents is preferred over single emulgent. The combination consists of a predominantly hydrophilic emulgent in the aqueous phase and a hydrophobic agent in the oily phase to form a complex film at the interface.

1.5.2 Multimolecular films

Hydrated lyophilic colloids form multimolecular films around globules of dispersed oil. Hydrated colloids do not cause any appreciable lowering of surface tension and their ability to form strong, coherent multimolecular films. Their tendency to increase the viscosity of the continuous phase enhances the stability of emulsion.

1.5.3 Solid particulate films

The emulgents forming particulate films are small solid particles that are wetted to some degree by both aqueous and non-aqueous liquid phases. They are concentrated at the interface where they produce a film around the dispersed globules thus preventing coalescence.

1.6 Method of preparation of nanoemulsion. [5]

Formulation of nanoemulsion includes active drug, additive and emulsifier. The various methods for the preparation of nanoemulsion include two methods:

(a) high-energy emulsification and

(b) low-energy emulsification.

The high-energy emulsification method includes

- high-energy stirring,
- ultrasonic emulsification,
- high-pressure homogenization,
- microfluidization,
- membrane emulsification

The low-energy emulsification method includes

- phase inversion temperature,
- emulsion inversion point, and
- spontaneous emulsification

Using a combined method, which includes the high-energy and low-energy emulsification, it is possible to prepare reverse nanoemulsion in a highly viscous system.

1.6.1.a Ultrasonic Emulsification:

Ultrasonic emulsification is very efficient in reducing droplet size. In ultrasonic emulsification, the energy is provided through sonotrodes called as sonicator probe. It contains piezoelectric quartz crystal which can expand and contract in response to alternating electric voltage. As the tip of sonicator contacts the liquid, it produces mechanical vibration and cavitation occurs. Cavitation is the formation and collapse of vapour cavities in liquid. Thus, ultrasound can be directly used to produce emulsion; it is mainly used in laboratories where emulsion droplet size as low as 0.2 micrometer can be obtained.

1.6.1.b High pressure homogenization:

The preparation of nanoemulsion requires high-pressure homogenization. This technique makes use of high-pressure homogenizer/piston homogenizer to produce nanoemulsion of extremely low particle size (up to 1 nm).

1.6.1.c Microfluidisation:

Microfluidization is a patented mixing technology, which makes use of a device called microfluidizer. This device uses high pressure which forces the drug product through the interaction chamber resulting in a very fine particle of submicron range. The process is repeated several times to obtain a desired particle size to produce uniform nanoemulsion.

1.6.2.a Phase inversion temperature:

This method involves change in phase by applying a higher temperature to a microemulsion

1.6.2.b Spontaneous emulsification:

It involves three steps: (a) preparation of homogeneous organic solution consisting of oil and lipophilic surfactant in water miscible solvent and hydrophilic surfactant, (b)stirring, o/w emulsion is formed, (c) the aqueous phase is removed by evaporation under reduced pressure.

1.7 EVALUATION OF NANOEMULSION [6]

1.7.1 Droplet size analysis

Droplet size analysis of nanoemulsion is measured by a diffusion method using a light-scattering, particle size-analyzer counter, LS 230. It is also measured by correlation spectroscopy that analyzes the fluctuation in scattering of light due to Brownian motion. Droplet size analysis of nanoemulsion can also be performed by transmission electron microscopy.

1.7.2 Viscosity determination

The viscosity of nanoemulsion is measured by using Brookfield-type rotary viscometer at different shear rates at different temperatures.

1.7.3 Dilution test

Dilution of a nanoemulsion either with oil or with water can reveal this type. The test is based on the fact that more of the continuous phase can be added into a nanoemulsion without causing the problem of its stability. Thus, an o/w nanoemulsion can be diluted with water and a w/o nanoemulsion can be diluted with oil.

1.7.4 Drug content

Preweighed nanoemulsion is extracted by dissolving in a suitable solvent, extract is analyzed by spectrophotometer or HPLC against standard solution of drug.

1.7.5 Polydispersity

It indicates the uniformity of droplet size in nanoemulsion. The higher the value of polydispersity, lower will be uniformity of droplet size of nanoemulsion. It can be defined as the ratio of standard deviation to mean droplet size. It is measured by a spectrophotometer.

1.7.6 Dye test

If a water-soluble dye is added in an o/w nanoemulsion the nanoemulsion takes up the colour uniformly. Conversely, if the emulsion is w/o type and the dye being soluble in water, the

emulsion takes up the colour only in the dispersed phase and the emulsion is not uniformly coloured. This can be revealed immediately by microscopic examination of the emulsion.

1.7.7 Refractive index

Refractive index of nanoemulsion is measured by Abbes refractometer.

1.7.8 pH

The pH of nanoemulsion can be measured by pH meter.

1.7.9 Zeta potential

Zeta potential is measured by an instrument known as Zeta PALS. It is used to measure the charge on the surface of droplet in nanoemulsion.

1.7.10 Fluorescence test

Many oils exhibit fluorescence when exposed to UV light. When a w/o nanoemulsion is exposed to a fluorescence light under a microscope, the entire field fluoresces. If the fluorescence is spotty, the nanoemulsion of o/w type.

1.7.11 Percentage transmittance

Percentage transmittance of nanoemulsion is measured by a UV-visible spectrophotometer.

1.7.12 Conductance measurement

The conductance of nanoemulsion is measured by a conductometer. In this test a pair of electrodes connected to a lamp and an electric source is dipped into an emulsion. If the emulsion is o/w type, water conducts the current and lamp gets lit due to passage of current between the electrodes. The lamp does not glow when the emulsion is w/o: oil being in external phase does not conduct the current.

1.7.13 Filter paper test

This is based on the fact that an o/w nanoemulsion will spread out rapidly when dropped onto filter paper. In contrast, a w/o nanoemulsion will migrate only slowly. This method should not be used for highly viscous creams.

1.7.14 In vitro Release Studies:

The drug release rate from the nanoemulsion was carried out using the USP dissolution paddle assembly. A weighed amount of nanoemulsion equivalent to 100 mg drug were dispersed in 900 ml of phosphate buffer 6.8 maintained at 37 ± 0.5°C and stirred at 100 rpm. At preselected time intervals one ml sample was withdrawn and replaced with equal amount of phosphate buffer 6.8. The collected samples were suitably diluted with phosphate buffer 6.8 and analyzed spectrophotometrically to determine the concentration of drug present in the dissolution medium. The dissolution studies were repeated using phosphate buffer pH 6.8 as dissolution medium.

1.8 CIRCULATORY SYSTEM [7,8]

Human body contain various systems such as skeletal system, muscular system, circulatory system, nervous system, respiratory system, digestive system, excretory system, endocrine system, reproductive system and lymphatic system. Circulatory system plays main role and functions to transport nutrients, gases, hormones and waste throughout the body.

The circulatory system is divided, for descriptive purpose into two main parts. The blood circulatory system consisting of the heart which acts as a pump and the blood vessels through which the blood circulates. The lymphatic system consists of lymph nodes and lymph vessels through which colorless lymph flows.

The two systems communicate with one another and are intimately associated. The lungs (pulmonary circulation) oxygen absorbed and releases carbon dioxide. The rest of the body (systemic circulation) supplies oxygen and nutrition to the cells and removing waste products. The circulatory system (shown in Fig: 1) is involved in hemostasis as it ensures continuous supply of blood to all body cells. Control of this system enables rapid response to change that affect delivery of adequate blood to the tissues. The supply of oxygen and nutrients to body cells becomes in adequate, hemostasis is threatened and tissue damage and death follows. Blood is

described as a connective tissue. It provides one of the means of communication between the cells of different parts of the body and the external environment.

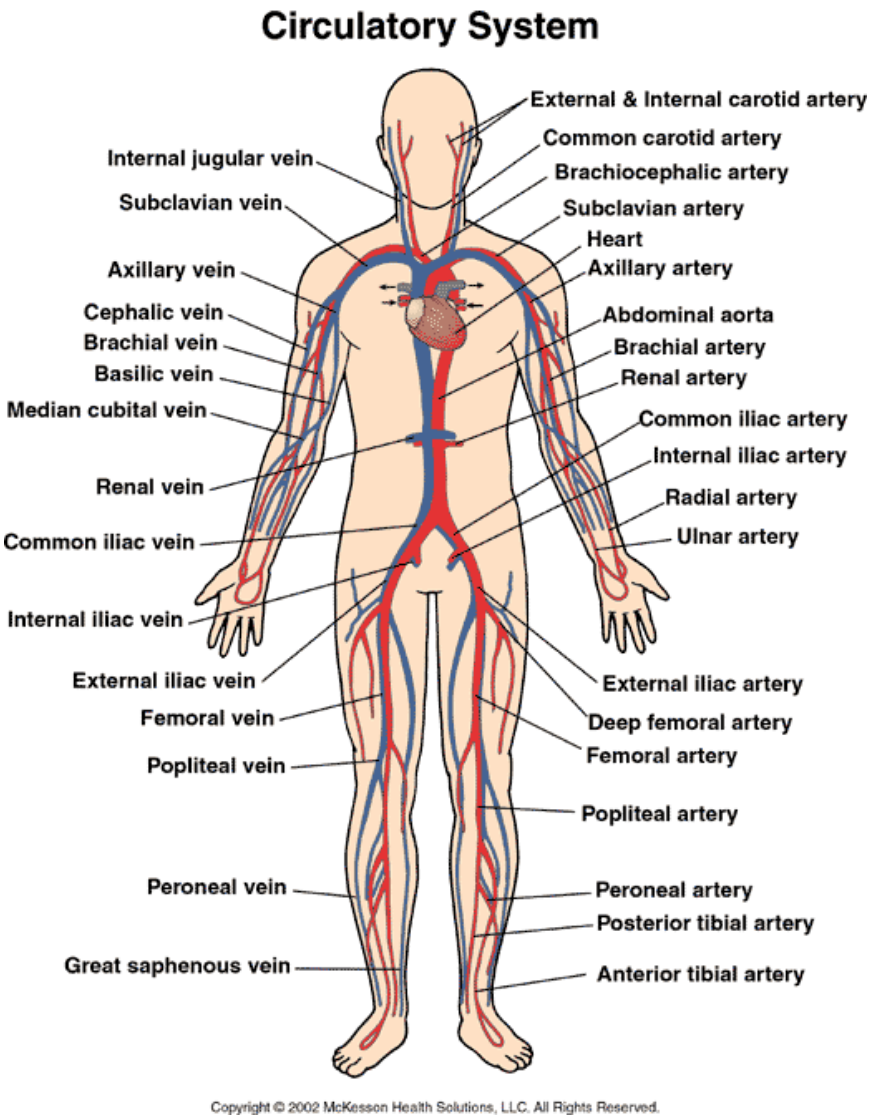


Fig: 1. The circulatory system.

Blood constitutes about 7 % of body weight. This proportion is less in woman and considerably greater in children. Blood in the blood vessel is always in motion. The flow is such that body cells have a fairly constant environment. Blood is composed of a straw colored transparent fluid called as plasma, in which different types of cells are suspended. Plasma constitutes about 55% and cells about 45% of blood volume. There are 3 types of cells such as

- Erythrocytes (or) red blood cells
- Leukocytes (or) white blood cells
- Thrombocytes (or) platelets.

All the blood cells originated from stem cells through several developmental stages before entering the blood. Erythrocytes are circular bi-concave non –nucleated disc with a diameter of about 7 micron (μ). They are formed in red bonr marrow present in the end s of the long bones and in flat and irregular bones. They pass through developmental stages before entering into circulation. Their life span is 120 days.

Hemoglobin is a complex protein consisting of globin and an iron part called as haem and is synthesized inside the developing erythrocytes in red bone marrow. Haemoglobin in erythrocytes combines with oxygen to form oxy-haemoglobin giving blood its characteristic red color.

Leukocytes have an important function in defending the body against microbes and other foreign materials. Leukocytes are the largest blood cells and they account for about 1% of the blood volume. They contain nuclei and some astemohave granules in their cytoplasm.

Platelets are very small non-nucleated discs, 2-4 μ in diameter, derived from the cytoplasm of megakaryocytes in red bone marrow. They contain a variety of substances that promote blood clotting which causes hemostasis.

Hemostasis is the process that maintains the blood within the blood vessels. It is a complicated but efficient mechanism interlocking responses of the blood vessels, the platelets, the coagulation factors and the fibrinolytic mechanism. Thrombogenesis coagulation of blood occurs in blood vessels that have not been injured, resulting in blockage of the concerned blood vessels leading to serious consequences.

1.9 Clot formation [9]

The formation of clot involves several steps. A thrombus is formed at sites of injury, such as a simple skin laceration or miscellaneous intravascular injury. Circulating platelets first adhere to the site of injury and a series of events occurs that allows activation of these platelets. Activated platelets then recruit additional platelets to the site of injury where they aggregate to stabilize the plug until the body can provide further stability to the clot. Increased platelet activity and

vascular injury simultaneously stimulated previously inactive coagulation factors which are always present and circulating in normal blood.

Activation of these clotting factors results in the initiation of the intrinsic pathway, the extrinsic pathway or both. The intrinsic and extrinsic coagulation factor interactions take place and then converge at the same point, named the common pathway , the coagulation cascade continues, initiating a series of events that ultimately leads to the formation of an insoluble, stabilized fibrin clot. The formation of a thrombus takes place approximately 12-16 second in a normal individual.

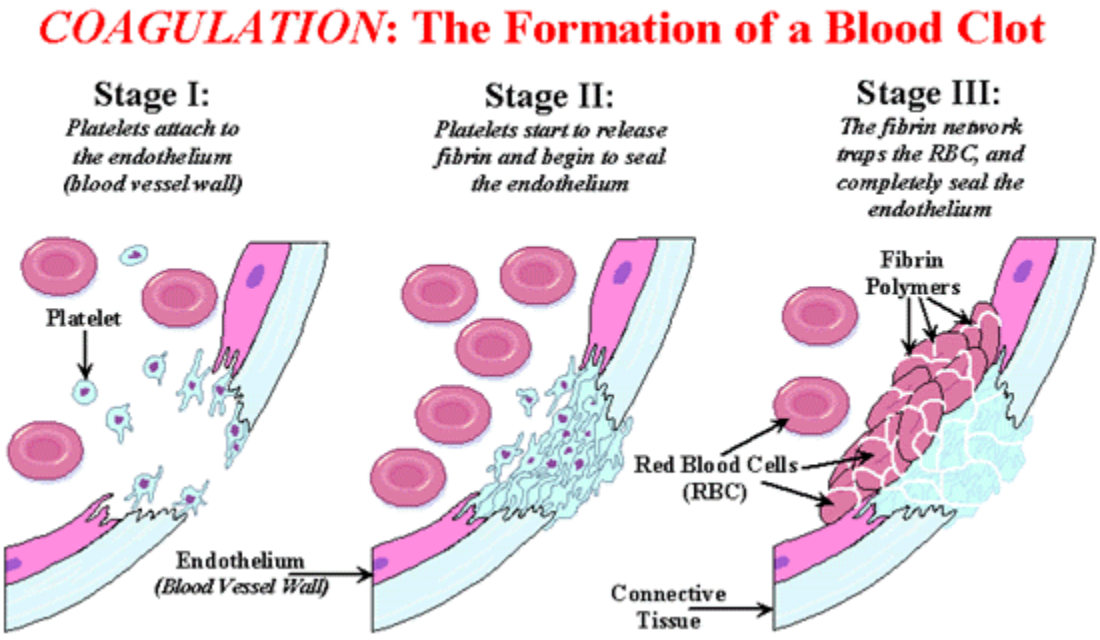


Fig: 2. Stages of blood clot formation.

1.10 Role of platelets and fibrin [10]

Platelets respond to the vascular trauma by an activation process which involves 3 steps

- Adhesion to the site of injury
- Release of intracellular granules
- Aggregation of the platelets.

Platelets first adhere to exposed collagen in the sub-endothelial layers of injured blood vessels, triggering the release of platelet granules containing chemical mediators, which promote platelet aggregation and the formation of a plug composed of the viscous contents of lysed platelets. This rapidly arrests bleeding. Local stimulation of the coagulation cascade by factors released from the injured tissue and platelets results in the formation of thrombin. Thrombin, in turn, catalyzes the conversion of fibrinogen to fibrin, which is incorporated into the plug. Subsequent cross-linking of the fibrin strands stabilizes the clot and forms a hemostasis plug .

1.11 Thrombus versus embolus [10]

A clot that adheres to a vessel wall is called a thrombus, while an intervascular clot that floats within the blood is termed as embolus. Thus, a detached thrombus becomes an embolus. Both thrombi and emboli are dangerous , since they may occlude blood vessels and deprive tissues of oxygen and nutrients. Arterial thrombosis most often involves medium sized vessels rendered thrombogenic by surface lesions of endothelial cells caused by atherosclerosis. In contrast, venous thrombosis is triggered by blood or in-appropriate activation of the coagulation cascade, often as a result of a defect in the normal defense hemostasis mechanism.

1.12 Types of thrombosis [11]

There are two distinct forms of thrombosis, each of which can be presented by several subtypes.

1.12.1 Venous thrombosis

Venous thrombosis is the formation of a thrombus within a vein. There are several diseases that can be classified under this category.

1.12.1.a Deep vein thrombosis

Deep vein thrombosis is the formation of a blood clot within a deep vein. It is most commonly affects leg veins such as the femoral vein.Three factors are important in the formation of a blood clot within a deep vein.These are the

1. Rate of blood flow
2. Thickness of the blood

3. Qualities of the vessel wall.

Classical signs of deep vein thrombosis are included swelling, pain, redness of the affected area.

1.12.1.b Portal vein thrombosis

Portal vein thrombosis is a form of venous thrombosis affecting the hepatic portal vein, which can lead to portal hypertension and reduction of the blood supply to the liver. It usually has a pathological cause such as pancreatitis, cirrhosis, diverticulitis or cholangiocarcinoma.

1.12.1.c Renal vein thrombosis

Renal vein thrombosis is the obstruction of the renal vein by a thrombus. This tends to lead to reduced drainage from the kidney. Anti coagulation therapy is the treatment of choice.

1.12.1.d Jugular vein thrombosis

Jugular vein is a condition that may occur due to infection, intravenous drug use or malignancy. Jugular vein thrombosis complications are including:

1. Systemic sepsis
2. Pulmonary embolism
3. papilloedema

Characterised by a sharp pain at the site of the vein, it is difficult to diagnose , because it can occur at random.

1.12.1.e Budd chiari syndrome

Budd chiari syndrome is the blockage of the hepatic vein or the inferior venacava. This form of thrombosis presents with abdominal pain , ascites and hepatomeagaly. Treatment varies between drug therapy and surgical intervention using of shunts.

1.12.1.f Paget schroetter disease

Paget schroetter disease is the obstruction of an upper extremity vein (such as the axillary vein or sub clavian vein) by the thrombus. The condition usually comes to light after vigorous exercise and usually presents in younger, otherwise healthy people. Men are affected more than women.

1.12.1.g Cerebral venous sinus thrombosis

Cerebral venous sinus thrombosis is a rare form of stroke which results from blockage of the dural venous sinuses by a thrombus. Symptoms are included headache, abnormal vision, any of the symptoms of stroke such as weakness of the face and limbs on one side of the body and seizures. The diagnosis is usually made with a computerized topography scan

.12.2 Arterial thrombosis

Arterial thrombosis is the formation of a thrombus within an artery. In most cases, arterial thrombosis follows rupture of atheroma and is therefore reffered to as "atherothrombosis". There are two diseases, which can be classified under this category:

1.12.2.a Stroke

A stroke is the rapid decline of brain function due to a disturbance in the supply of blood to the brain. This can be due to ischemia, thrombus, embolus (a lodged particle) or haemorrhage (a bleeding). In thrombotic stroke, a thrombus (a blood clot) usually forms around atherosclerotic plaques. Since blockage of the artery is gradual, onset of symptomatic thrombotic strokes is slower. Thrombotic stroke can be divided into two categories :

1. Large vessel disease
2. Small vessel disease

The former affects vessels such as the internal carotids, vertebral and the circle of willis. The later can affect the smaller vessels such as the branches of the circle of willis.

1.12.2.b Myocardial infraction

Myocardial infraction is caused by an infract (death of tissue due to ischemia), often due to the obstruction of the coronary artery by a thrombus. Myocardial infraction can quickly become fatal if emergency medical treatment is not received promptly. If diagnosed within 12 hour (hr) of the initial episode (attack) then thrombolytic therapy is initiated.

1.13 Symptoms (www. Emedicine.com)

Venous clot do not allow blood to return to the heart. Most often occurring in the legs or arms, symptoms include as follows:

- Swelling
- Warm
- Redness
- pain

Arterial lots do not allow blood to get to the affected area. Body tissue that is deprived blood and oxygen begins to die and becomes ischemic.

- Pain is the initial symptom of the ischemic, or oxygen deprivation due to loss of the blood supply.
- Other symptoms depend upon the location of the clot often the effect will be a loss of function. Heart attack and stroke are self –explanatory.
- In an arm or leg, in addition to pain, the limb may appear white and weakness, loss of sensation , or paralysis may occur.
- If the blood supply is lost to an area of the bowel, in addition to intense pain, there may be bloody diarrhoea.

1.14 Diagnosis [13]

The initial step in making the diagnosis of a blood clot is obtaining a patient history. The blood clot a does not cause any problem but the location of the blood clot and its effect on blood flow that causes symptoms and signs.

If a blood clot or thrombus is a consideration, the history may expand to explore risk factors or situations that might put the patient at risk for forming a clot. Venous blood clots often develop slowly with a gradual onset of swelling, pain and discolouration. Symptoms of a venous thrombus will often progress over hours. Arterial thrombi occurs as an acute event. Tissues need oxygen immediately and the loss of blood supply creates a situation in which symptoms begin immediately.

There may be symptoms that precede the acute artery blockage that may be the warnings signs of the potential future complete occlusion of the blood vessel.

- Patients with an acute heart attack (myocardial infraction) may experience angina in the days and weeks prior to the heart attack.
- Patients with peripheral artery disease may have pain with walking (claudication) and a transient ischemia attack, mini stroke may precede a stroke.

Physical examination can assist in providing additional information that may increase the suspicion for a blood clot.

1.14.1 Venous thrombi:

It may cause swelling of an extremity. It may be red, warm and tender sometimes the appearance is difficult to distinguish from cellulitis or an infection of extremity. If there is a concern about a pulmonary embolus, the clinician may examine the lungs, listening to the abnormal sound caused by an area of inflamed lung tissue.

1.14.2 Arterial thrombi:

The symptoms are much more dramatic. If a leg or arm is involved, the tissue may be white because of the lack of the blood supply. As well, it may be cool to touch and there may be a loss of sensation and movement.

The patient may be writhing in pain. Arterial thrombus is all the cause of heart attack(myocardial infraction) and stroke(cerebro-vascular accident) and their associated symptoms.

1.15. Drugs for the treatment of thrombosis:

1.15.1 Anti-coagulation drugs [13]

1.15.1.a Heparin

Heparin (structure shown in Fig: 3) is a polysaccharide consisting of a chain of repeating sulphated disaccharide units of 60,000 – 100000 daltons. It is found in secretory granules of mast cells attached to a core protein. Heparin fragments reffered to as low molecular weight heparin

are used increasingly in the place un fractionated heparin. Heparin inhibits coagulation both in-vivo and in-vitro by activating antithrombin III. Antithrombin III inhibits thrombin and other serine protease by binding to the serine site. Low molecular weight heparin increases the action of antithrombin III and factor Xa but not its action on thrombin, since the molecules are too small to bind to both enzymes and inhibitor essential for inhibition of thrombin but not for that of factor Xa. Heparin in higher doses interferes with platelets aggregation and prolongs bleeding time.

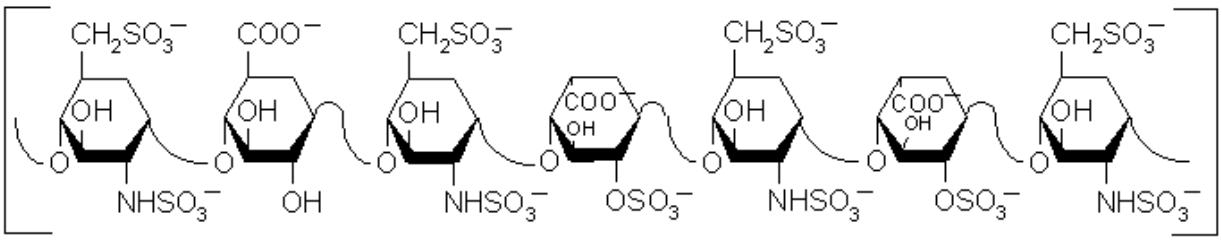


Fig: 3. Structure of Heparin

The standard intra venous schedule is usually 5,000 units loading intravenous followed by 1,000 – 1,500 units per hour infusion by an infusion pump to give a total of 20,000 – 40,000 units per day to achieve an activated partial thromboplastin time of 1.5 – 2 times the control. Intermittent dosing can be done with an initial intravenous every 4 – 6 hour. Subcutaneous heparin in the dose of 5,000 units every 8 - 12 hour prevents post operative thrombo embolism.

The un-fractionated heparin is composed of sulfated polysaccharide molecules with a molecular weight range from 5,000 – 30,000 daltons and an average molecular weight of 12,000 – 15,000 daltons. In the treatment of deep vein thrombosis, it has been found effective in a dose of 5,000 units once daily.

1.15.1.b Warfarin

Warfarin (structure shown in Fig: 4) a coumarin derivative, inhibits clotting by limiting hepatic production of the biologically active vitamin K-dependent clotting factors (activated factors II, VII, IX, X).Normally the precursors of these factors under go a carboxylation rection to be coverted to their activated forms. Warfarin , as a vitamin K antagonist with this reaction.

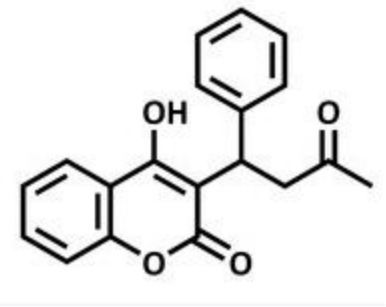


Fig: 4. Structure of Warfarin.

The reduction in the amount and activity of these factors produces the anticoagulant response. However warfarin also interferes with production of the body's natural anticoagulants, protein C, protein S. Peak plasma level is achieved between 2 – 8 hr after oral administration. They are highly bound to plasma protein (90 – 99%) principally to albumin. Sensitivity of anticoagulants varies from patient to patient and recquires individual adjustment of dose.

Dose adjustments are done to keep a pro-thrombin time between 1.5 – 2 times and international normalized ratio 2 – 3 times of control. Loading dose of 10 –15 mg is followed by 1 – 10 mg per day maintenence dose.

1.15.2 Anti-platelet drugs [14]

The major role of antiplatelet drugs in clinical practice is to prevent the adverse clinical sequelae of thrombosis in atherosclerotic arteries to the heart (acute coronary syndrome),brain (ischaemic stroke), and limbs (inter-mittent claudication and rest pain) and thrombosis of stagnant blood in veins (venous thromboembolism) and heart chambers (atria fibrillation, heart failure). Dose and route of administration of anti-platelets drugs are in the Table no:1.

1.15.2.a Aspirin

Aspirin is chemically named as 2-acetoxybenzoic acid. It irreversibly inhibits prostaglandin H synthetase (cyclooxygenase-1) in the platelets and megakaryocytes and thereby blocks the formation of thromboxane A2(a potent vasoconstrictor and platelet aggregant). It is only parent form acetylsalicylic acid which has any significant effect on platelet function. Because platelets are unable to regenerate cyclooxygenase, the immediate anti-thrombotic effect of aspirin remains for the lifespan of the platelet (8-10 days). As, after stopping the aspirin therapy normal

hemostasis may be regained when about 20% of platelets have normal cyclooxygenase activity,daily aspirin intake is recommended.

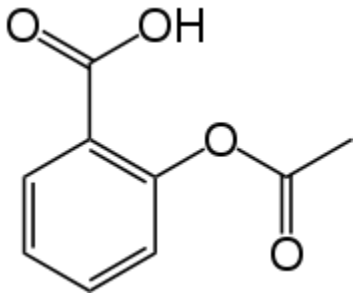


Fig: 5. Structure of Aspirin.

1.15.2.b Clopidogrel and ticlopidine

The thienopyridine derivatives clopidogrel (structure shown in Fig: 6) and ticlopidine are metabolized in the liver to active compounds which covalently bind to the adenosine phosphate receptor on platelets and dramatically reduce platelet activation.

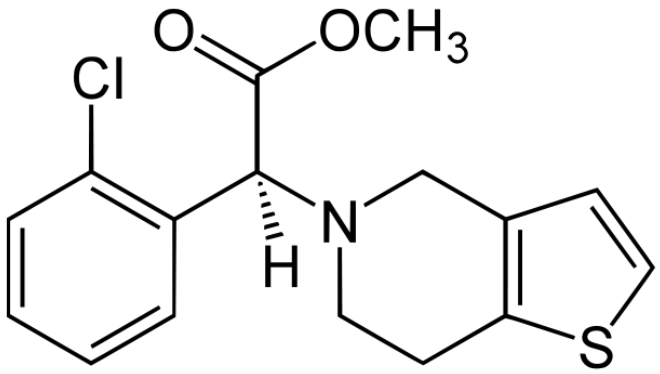


Fig: 6. Structure of clopidogrel.

1.15.2.c Dipyridamole

Dipyridamole (structure shown Fig:7) inhibits phosphodiesterase which inactivates cyclic adenosine monophosphate (AMP). Increased intraplatelet concentrations of cyclic AMP reduce the activation of cytoplasmic second messengers. Dipyridamole also stimulates prostacyclin release and inhibits thromboxane A2 formation. Because the effect is short-lasting , repeated dosing or slow-release preparations are recquired to inhibit platelet function for 24 hr.

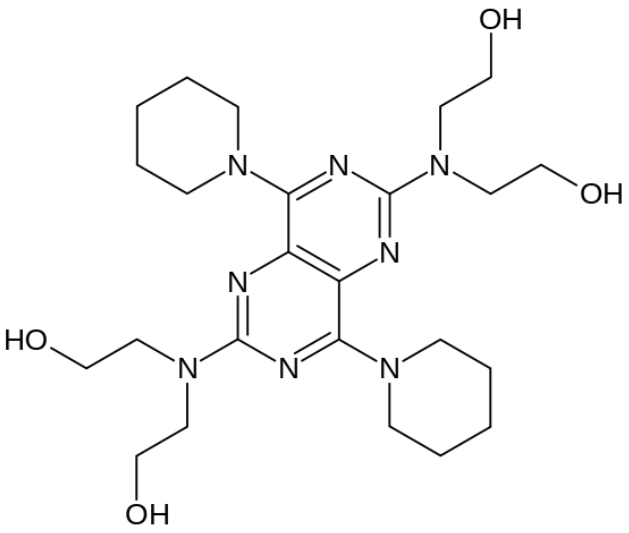


Fig: 7. Structure of Dipyridamole.

1.15.2.d Glycoprotein IIb/IIIa receptor blockers.

Glycoprotein IIb/IIIamreceptor antagonists block the final common pathway for platelet aggregation. Abciximab is a humanized mouse antibody fragment with a high binding affinity for the glycoprotein IIb/IIIa receptor. Tirofiban (a non-peptide derivative of tyrosine) (structure shown in fig:8) mimic part of the structure of fibrinogen that interacts with the glycoprotein IIb/IIIa receptor and thus compete with ligand binding of fibrinogen to the glycoprotein IIb/IIIa receptor.

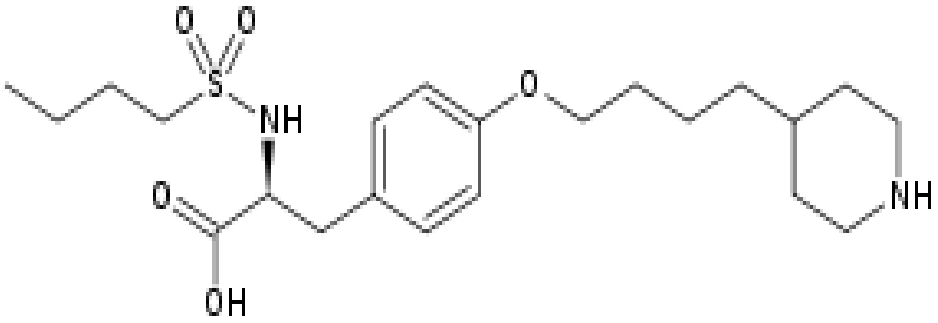


Fig: 8. Structure of Tigrofiban.

Table: 1. Dose and route of administration of anti-platelet drugs.

Drug name	Dose	Route
Aspirin	Load: 160 mg Maintenance 75-150mg once daily.	Oral
Clopidogrel	Load: 300mg Maintenance 75 mg once daily	Oral
Ticlopidine	250 mg twice daily	Oral
Dipyrimdamole	200 mg twice daily	Oral
Abciximab	Bolus 250 (µg/kg) Infusion 0.125µg/kg/min	Intravenous 30 min Infusion 24-72 hr
Tirogiban	Bolus 0.4- 0.6 µg/kg Infusion 0.10-0.15µg/kg/min	Intravenous 1.2-1.6 hr Infusion 24-72 hr.

1.15.3 Thrombolytic agents

Thrombolytic drug dissolves blood clots by activating plasminogen, which forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots. Because of these actions, thrombolytic drugs are also called as "plasminogen activators" and "fibrinolytic drugs". Thrombolytic agents are used to lyse already formed blood clots in clinical settings, where ischemia may be fatal (acute myocardial infraction, pulmonary embolism, ischemic stroke and arterial thrombosis). Very precise indications rule the use of these drugs,which are not free from serious side effects (bleeding).

1.15.3.a Tissue - type plasminogen activator

Tissue-type plasminogen activator is a serine protease originally derived from cultured human melanoma cells, but it is now obtained in therapeutic quantities as a product of recombinant de-oxyribo nucleic acid (DNA) technology. Tissue- type plasminogen has a low affinity for free plasminogen, but it rapidly activates plasminogen bound to fibrin in a thrombus or a hemostasis plug. Thus, tissue-type plasminogen is said to be fibrin selective and has the advantage of lysing only the fibrin, without unwanted degradation of other proteins, notably fibrinogen. This contrasts with urokinase and streptokinase, which act on free plasminogen and induce a thrombolytic state. This advantage seems to be realized at low doses of tissue-type plasminogen, but at high doses a thrombolytic state is induced with the risk of hemorrhage.

1.15.3.b Streptokinase

Streptokinase (structure shown in Fig: 9) is an extracellular protein derived from purified culture broth of group C β-hemolytic streptococci. Streptokinase has no enzymatic activity; instead it forms an active complex with plasminogen, which then converts uncomplexed plasminogen to the active enzyme plasmin. In addition to the hydrolysis of fibrin plugs, the complex also catalyses the degradation of fibrinogen as well as clotting factors V and VII. It has half-life of 23 min and for mocardical infraction. It is given in a dose of 7.5 -15 lakh units over 1 hr by intravenous infusion.

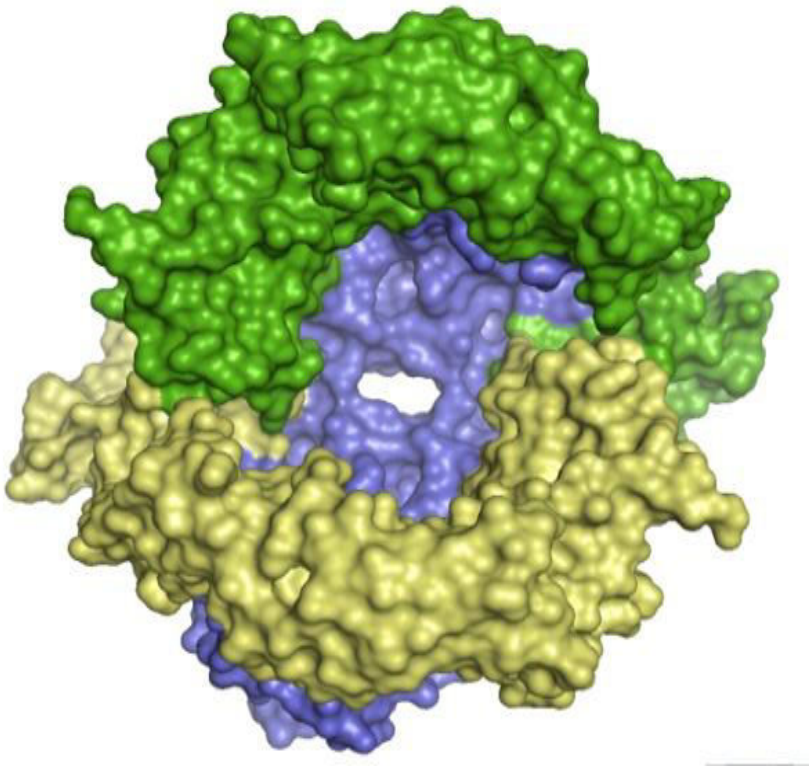


Fig:9. Structure of Streptokinase.

1.15.3. c Urokinase

Urokinase is an enzyme capable of directly degrading both fibrin and fibrinogen. Urokinase was originally isolated from human urine, but it is now obtained from cultures of human fetal renal cells. Urokinase is more expensive than streptokinase and is usually employed in patients who are sensitive to streptokinase. It is not a foreign protein, therefore non-antigenic. For myocardial infraction, it is given in a dose of 2.5 lakhs units intravenous over a 10 min period followed by 5 lakhs units over that 60min.

AIM AND OBJECTIVE

Aim and objective of the study

Thrombosis is the formation of an unwanted clot within a blood vessel or heart. It is the most common abnormality of hemostasis. Thrombolytic drugs are used to lyses blood clot. Blood clot can occur in any vascular bed, however, when they occur in coronary, cerebral, or pulmonary vessels, they can be immediately life threatening. Therefore, it is important to diagnose and treat rapidly thrombosis.

On this regard, formulation of a natural thrombolytic drug with advanced technique of drug delivery for a safe and effective administration is the aim of this study.

The main objective of the study is to

- Prepare an extract of the dried aerial parts of the plant *Bauhinia racemosa.*
- Acute toxicity study of the extract.
- Determination of thrombolytic effect of the extract.
- Preparation of the nanoemulsion of the plant extract.
- Characterization of the nanoemulsion.
- Stability study of nanoemulsion.

PLAN OF WORK

- Extraction of the dried leaves of *Bauhinia racemosa.*
- Acute toxicity study of the extract.
    - Gross behavior study.
    - Body weight analysis.
    - Hematological parameters.
    - Bio-chemical parameters.
    - Gross necropsy and histopathological studies.
- Determination of thrombolytic activity of the extract.
- Formulation of extract loaded nanoemulsion.
- Evaluation and characterization of the prepared nanoemulsion.
    - Particle size distribution.
    - Zeta potential.
    - Particle morphology study.
    - In-vitro release study.
- Stability study of the prepared nanoemulsion.

LITERATURE REVIEW

3.1 Literature review for extraction method for *Bauhinia racemosa* leaves

Sharanabasappa et al, (2007) conducted phytochemical study on *Bauhinia racemosa.* The leaves of the plant were shade dried, powdered, and then extracted with ethanol using soxhlet apparatus.

Sunil nirmal et al, (2011) studied the antihistaminic effect of *Bauhinia racemosa* leaves in swiss albino mice. For this purpose the fresh leaves of the plant were shade dried, crushed to produce a coarse powder and subjected to extraction in a soxhlet apparatus using ethanol. The yield was reported to be 15.3% w/w.

Pramila et al, (2014) conducted a study on biological activity of aqueous extract of some medicinal plants where in the fresh leaves of *Bauhinia racemosa* were collected shade dried powdered coarsely and then extracted with ethanol by using soxhlet apparatus.

Ghumare pramila et al, (2014) carried out the preliminary phytochemical screening and anti bacterial activity of *Bauhinia racemosa* leaves. Aqueous, ethanol, chloroform, acetone and petroleum ether extracts of the leaves were prepared and its antibacterial activity was studied. It was concluded that the ethanol and acetone extracts showed good anti bacterial activity when compared to the other extracts.

Kesavan et al, (2011) studied the pharmacological properties of *Bauhinia racemosa* leaf extract. The ethanol extract showed significant anti-diarrhoeal and anti inflammatory activity. The results obatained was comparable to the standard drug activity used.

3.2 Literature review for in-vitro thrombolytic activity testing

Mohammad mamun ur Rashid et al, (2014) carried out the in-vitro thrombolytic activity of methanolic extract of *Protium serratum* leaves where in the degree of lysis of the clot was found to be 59.65%, while the standard streptokinase and water used as positive and negative controls demonstrated 72.835 and 2.725 % of clot lysis respectively.

Mohammad Sekender Ali et al, (2013) studied the thrombolytic activity of methanolic extract of the leaves of *Adiantum philippense* by clot disruption method where the extract shows 12.86% and standard streptokinase shows 30.86% of clot lysis respectively.

Md. Reyad-ul-Ferdous et al,(2014) carried out the thrombolytic activity with the methanolic extract of *Bauhinia acuminate* leaves and compared it to the standard drug streptokinase. It showed significant thrombolytic effect which was about 10.058%.

Mohammad Shahadat Hossain et al, (2012) studied the in-vitro thrombolytic activity of ethanolic extract of *Swertia chirata* using the in-vitro clot lysis method.The crude ethanol extract was found to have a significant activity that showed a maximum effect of 40.38% while standard streptokinase showed 69.35%.

Pushplata chougule et al, (2014) studied the in-vitro thrombolytic activity of ethanolic extract of leaves of *Aegle marmelos.* Here the study of thrombolytic activity of aegle marmalos was carried out by using a simple and quick in-vitro clot lysis method which exhibited a maximum clot lysis of 84% at 800 µg/ml of concentration in 90 mins of incubation at 37 C. Various concentrations of leaf extract i.e 200 µg/ml, 400 µg/ml, 800 µg/ml were tested at time intervals of 90 mins and incubation at 37° C for observing maximum clot lysis. The result findings indicated that concentration of leaf extract enhanced the percentage of clot lysis in dose dependent manner. Streptokinase and water were used as positive and negative control that showed clot lysis of 89% and 2% in 90 mins of incubation at 37° C respectively.

Shah Md. Shahik et al, (2014) studied the in-vitro thrombolytic activity of *Mentha spicata, Mentha virdis, Mentha arvensis.* The study was carried out to check the clot lysis effect of the three plants where in streptokinase was used as a positive control and water as a negative control. The methanolic extract of the plant showed good thrombolytic acivity when compared with the ethanol, chloroform, acetone extract of the plants.

Ramesh Londonkar et al, (2014) carried out the evaluation of in-vitro thrombolytic activity and cytotoxicity potential of *Typha angustifolia* leaf extracts. The methanol, aqueous and chloroform extracts were evaluated for the clot lysis effect. The extracts showed 58±2.32%, 51.76±2.5%,18±1.84% of clot lysis, where as the positive control streptokinase 79.6±1.10% and negative control water 2.44±0.62% of clotlysis respectively.

3.3 Literature review for toxicity studies.

Guimaraes et al, (2014) conducted a study on assessment of acute toxicity study of ethanolic extract of *Lychnophora pinaster.* Acute toxicity of the crude ethanolic extract was evaluated by administration of the extract by oral route to male and female Swiss mice. A single extract dose of 125, 250 or 500 mg/kg was administered and the effects on spontaneous locomotor activity, exploratory behavior, muscle strength, body weight, food and water consumption, relative organ weight, histology, as well as hematological and biochemical parameters were evaluated. The three doses administered to the animals did not cause muscle tone alterations, but doses of 250 and 500 mg/kg induced a significant inhibition of the spontaneous locomotor activity and exploratory behavior of the animals in open-field test. There was no alteration to hematological parameters and consumption of water and food, body weight variation and organs relative weight. Changes were observed in AST and ALT during assessment of biochemical parameters. The histopathological evaluation showed that the extract provoked cellular alterations, such as vacuolar degeneration and inflammation in kidneys and liver at all doses. Liver morphometric analyses of male and female mice showed that the extract did not have dose-dependent effects. Although females showed a significant increase in inflammatory cells, the effect was not dose-dependent.

R.K. Patel et al, (2012) carried out acute and sub acute oral toxicity evaluation of *Benincasa hispida* extract in rodents. The toxicity studies were carried out a 50% aqueous ethanolic extract of *Benincasa hispida* (*B. hispida*)in rodents.The acute toxicity study, *B. hispida* was found to be well tolerated upto 2000mg/kg, produced neither mortality nor in behavior in mice. In subacute toxicity study, *B.hispida* at dose level of 200 and 400 mg/kg did not produce any significant difference in their body weight, food and water intake when compared to vehicle treated rats. It also showed no significant alteration in hematological and biochemical parameters in experimental groups of rats apart from a decrease in aspatate transaminase, alanine transaminase and alkaline phosphate content at the dose of 400 mg/kg. Histopathological study revealed normal architecture of kidney and liver of *B. hispida* treated rats. These results demonstrated that there is a wide margin of safety for the therapeutic use of *B. hispida* and further corroborated the traditional use of this extract as an anti hepatocarcinogenic agent.

Lalitha et al, (2013) carried out the acute oral toxicity studies of *Anacyclus pyrethrum* DC roots in albiono rats. The present study was aimed to determine LD50 and to establish the safety of different solvents likewise petroleum ether, chloroform, ethyl acetate, acetone, ethanol, water extracts of *Anacyclus pyrethrum* DC (Asteraceae) root by acute oral toxicity study in female rats as per OECD guideline 423. Rats were sequentially administered all the extracts in single dosages of 175, 550, and 2000 mg/kg of body weight. All the animals were individually studied for mortality, wellness parameters and body weight for 14 days. No mortality and no significant changes were observed in body weight and wellness parameters at 175, 550 and 2000 mg/kg body wt. doses, which reveal the safety of these extracts in the doses up to 2000 mg/kg body weight. Conclusively, LD50 value of *A. pyrethrum* DC root extracts was found to be more than 2000 mg/kg body weight.

Eugine et al, (2013) did the acute toxicity studies of andrographoloide. The present study has been designed with the objective to examine the andrographolide (isolated form A.paniculata) in order to evaluate its acute toxicities in experimental animal swiss albino mice. In acute toxicity studies the andrographolide 2000 mg/kg body weight was administered orally, observed after dosing and also observed for 14 days. Andrographolide effects on body weight, gross necropsy, hematological parameters, and biochemical parameters were studied. No significant variation in the body weight and organ weight between the control and the treated group was observed after single administration of andrographolide. Hematological and biochemical parameters of the control and the treated group revealed no toxic effect of the Andrographolide. No mortality was observed during 14 days study. From this study it may non-toxic through the oral route upto 2000mg/kg body weight dose level.

Abrar hussain mir et al, (2013) carried out an acute oral toxicity study of methanolic extract from *Tridex procumbens* in Sprague Dawley's Rats as per OECD guidelines 423.The present study has been under taken to study the adverse or hazardous effects of methanolic extract from Tridex procumbens, dissolved in Dimethyl sulphoxide (DMSO) & accordingly to determine the LD50, to establish the safety of methanolic extract of Tridex procumbens in SD Rats as per OECD guidelines 423. All the Rats were sequentially administered orally the methanolic extract first in a single dosage of 2000 mg/kg body weight. All the animals were observed for mortality, wellness parameters and body weight for 14 days and due to some morbidity and mortality the

experiment was again performed at same dosage and same results were observed. Then decrease in the dosage to 300 mg/kg body weight was performed and accordingly observed as per OECD Guidelines 423. No mortality or any significant change was observed at 300 mg/kg body weight, however at 2000 mg/kg body weight dose the mortality rate was 2/3. Conclusively indicates the LD50 value of Tridex procumbens methanolic extract to be less than 2000 mg/kg body weight and more than 300 mg/kg body weight (LD50 ≥ 300 mg/kg body weight, but < 2000 mg/kg body weight).

Oyemitan et al, (2013) carried out the acute toxicity , antinociceptive and anti-inflammatory activity of the essential oils of fresh fruits of *Piper guineense* Schum and Thonn in rodents. The study investigated the antiniciceptive and anti-inflammatory effect of the plant fruit volatile component and determines its acute toxicity profile in rodents in an attempt to rationalize the use of the plant in folkloric medicine. Essential oil of fresh fruits of P.guineense obtained by hydrodistillation was emulsified with Tween 80 and was evaluated for acute toxicity test (LD_{50}) through the oral (p.o) and intraperitoneal (i.p) routes in mice. The (LD_{50}) values obtained were 693 mg/kg (i.p) and 1265 mg/kg (p.o). This study shows that the essential oil of P.guineense was moderately toxic.

Satish et al, (2013) carried out the acute toxicity study of *Murraya koenigii*. Acute toxicity study of Murraya koenigii was carried out against Swiss albino mice. Behavioural assessment and LD 50 study was carried out. Results of present study shows that no mortality was observed at the highest dose level. The acute toxicity studies of crude powder (MCR) and methanol extract (MME) of Curry Leaves (Murraya koinigii) leave showed that they did not possess any toxic effect at the studied dose levels and are safe till the dose level of 9000 mg/ kg.

3.4 Literature review for formulation of nanoemulsion

Amudha et al, (2014) prepared and evaluated the self nanoemulsion containing *Eclipta alba* crude extract. The emulsion was formulated by homogenization technique and evaluated for SEM, drug content and in-vitro diffusion study. From the results it was concluded that the self nano emulsion containing particles was at nanometric range and also the release characteristic studies shows an immediate better release pattern, so the self nano emulsion may enhance the solubility and bioavailability of herbal drugs.

Merrie Natalia et al, (2014) studied the physical stability and antibacterial activity of *Nigella sativa* oil nanoemulsion gel. This *Nigella sativa* oil also called as black cumin oil was formulated into nanoemulsion gel in various concentrations of 5%, 7%, 9%. Physical stability test was conducted by recording the effect of storage at room temperature, high and low temperature respectively, a centrifugation test and a cycling test. The results showed a yellow orange colored translucent nanoemulsion gel was prepared with no phase separation and with a globule size of 1 micrometer. The nanoemulsion gels were stable at room temperature and low temperature.

Hayder Kadhim Drais et al, (2015) carried out the formulation and characterization of carvedilol nano emulsion oral liquid dosage form. The formulation components were chosen according to the solubility study. The diagrams of pseudo-ternary phase were made using the aqueous phase titration method. It was characterized by a low globule size range, acceptable low viscosity, low polydispersity index, higher zeta potential, good Ph value, efficient electroconductivity, classy percent of light transmittance, higher percentage drug content.

Meenakshi sinha et al, (2015) had under taken the preparation and characterization of nanoemulsion based on *carica papaya* seed oil. Crude extract from the seeds of carica papaya induce variable responses depending on dose, duration and route of administration in laboratory animals. Nanoemulsion optimized formula were found at 75% water, 9% surfactant (tween 80), 1% co-surfactant (span80) and 10% oil. The formulation was then characterized for droplet size, pH, TEM, zeta potential amd conductivity, in-vitro drug release.

Caio P. Fernandes et al, (2015) had carried out the study on development and characterization of evening primrose *(Oenothera biennis)* oil nanoemulsion. Here the required hydroprophile-Lipophile balance of evening primrose seeds oil was found to be 12. A stable nanoemulsion was formulated and characterized for mean droplet size, polydispersity index.

Mukesh et al, (2014) had worked on the topic development and characterization of *Boswellia serrata* plant extract loaded nanoemulsion mixtures of for the treatment of anti inflammatory disorders. Here the spray dried *Boswellia serrata* extract was developed and charcterized to compare it in-vitro diffusion with reference formulation. Pseudo-ternary phase diagram was constructed by aqueous titration method, of which the evaluation method was improved for solubility, droplet size and transmittance. The optimized nanoemulsion consisted of isopropyl

myristate, tween 80 and ethanol (2:8:10 w/w) and it remained stable for storing at 40°C, 4°C, 25°C for atleast six months. The formulation was then evaluated for droplet size, zeta potential, structure morphology by TEM.

Li Shupeng et al, (2011) carried out the work on preparation of w/o Astragulus polysaccharide Echinacea extract based nano emulsion and its amplified immune response. The formulation was optimized by the range of nanoemulsion zone in phase diagrams and the observations of naked eye. Its physicochemical properties were investigated including morphology, particle size distribution, pH, viscosity and stability. The formulation consisted of Tween 80, Span 80, paraffin oil, ethanol and water (with the volume ratio of 3:1:1.5:0.5:1) which was light yellow fluid. The particles were spherical under TEM, the viscosity and pH were 4.60s and 6.71 respectively.

Chutamas Uthumpa et al, (2013) developed a nanoemulsion formulation of ginger extract. The formulation was prepared from a mixture of oil phase (coconut oil and squalene), cremophor ELP, acetone and ginger extract by spontaneous emulsification method. Effect of coconut oil and squalene mass ratio on nanoemulsion formulations were evaluated further for the optimization of the system characterized by droplet size, polydispersibility index, zeta potential and Oswald ripening. Stability studies at 4°C and ambient temperatures for 3 months were performed and the formulation was found to be stable.

Kumar et al, (2014) carried out a study on enhanced intestinal permeability of *Tinosporia cardifolia* stem extract through nanoemulsion formulation. The formulation was prepared by sonication-solvent evaporation method and optimized for encapsulation efficiency, release kinetics as well as stability parameters by pseudo-ternary phase diagram. Clove oil was used as a lipid phase while Span 80 and Tween 20 were used as surfactant. The optimized formulation consisted of clove oil, span 80, Tween 20 and ethanol (7.7:4:0.5:21w/w) and 83.8% of the extract. The nanoemulsion remained stable during the 3 months of the study.

Heni Rachmawati et al, (2014) carried out the work on curcumin nanoemulsion for transdermal application: formulation and evaluation where in a nanoemulsion was formed by the self nano-emulsification method, using an oil phase of glyceryl monooleate, cremaphor RH-40, poly ethylene glycol 400. Evaluation of the nanoemulsion include particle size analysis,

polydispersibility index, zeta potential, physical stability, raman spectrum and morphology. The spontaneously formed stable nanoemulsion had a capacity of 350 mg of curcumin / 10 gram of oil phase. Overall, the developed nanoemulsion system not only had an improved curcumin permeability but also protected the curcumin from chemical degradation.

Marziyeh Khatibzadeh et al,(2014) had done their work on preparation of *Azardirachta indica* (neem) seed oil emulsion. In this study an attempt was made to prepare water based nanoemulsions from Neem seed oil using Tween 20 as a surfactant. Ultrasonic waves were used for applying shear to micro-scale droplets and altering them to nano-scale. Nanoemulsion with Neem oil and surfactant ratio of 1:4.5 which was sonicated for 45 minutes contained 50.21 nm droplets.

DRUG PROFILE

5.1 Plant profile of *Bauhinia racemosa* [15]

Vernacular names:

Hindi - katmauli

Tamil - atti

Malayalam - arampalli, mandaram

Telugu - tella arecettu

Sanskrit - yugmapatra

Urdu - Gul-e-anehal

Taxonomical classification

Kingdom - Plantae

Division - Angiosperma

Class - Dicotyledonae

Order - Fabales

Family - Caesalpiniaceae

Genus - *Bauhinia*

Species - *racemosa*

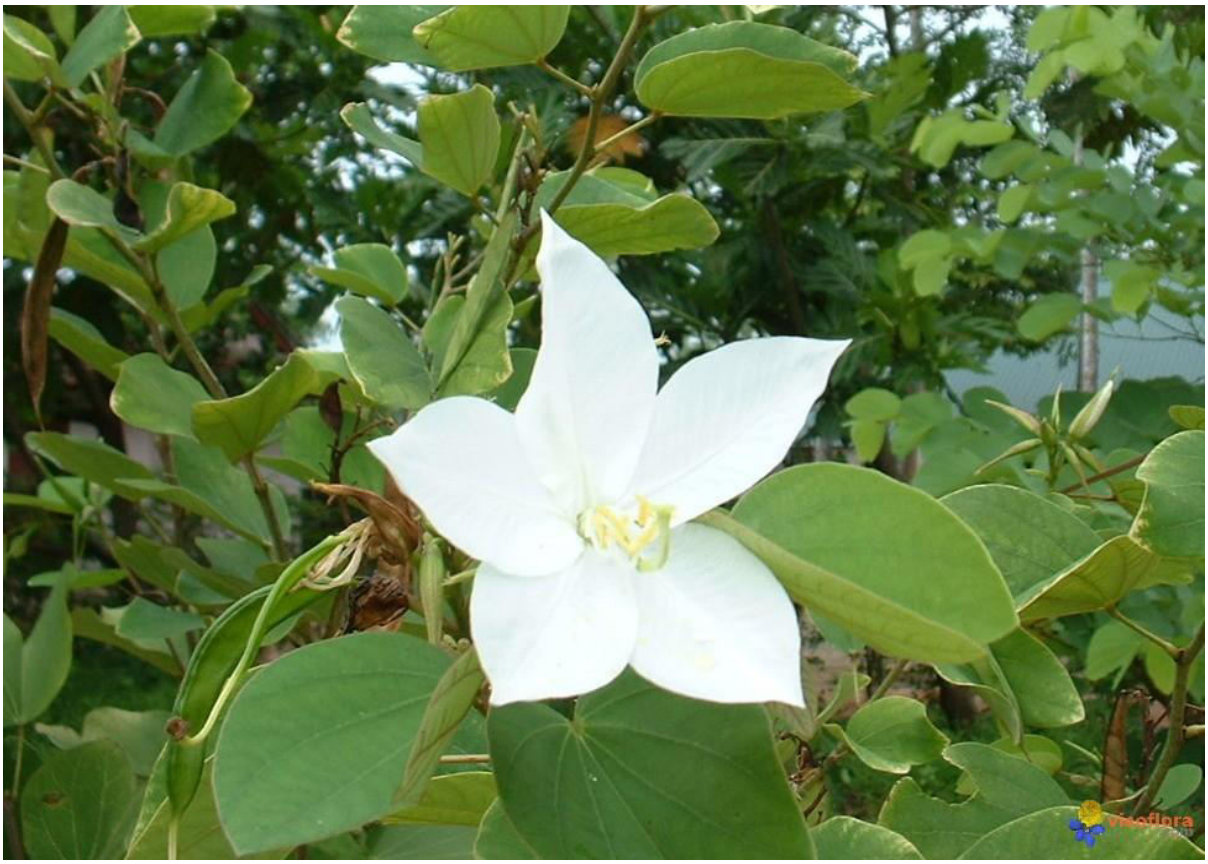


Fig 10. *Bauhinia racemosa plant*

Botanical description

Bauhinia racemosa is a small crocked tree with drooping branches, going up to 3-5 meters tall. Leaves are shaped like cow's hooves (the typical *Bauhinia* like shape). They are broader than long 2-5*3-6 cms. Small flowers are borne in loose racemes 5-10 cm long. Flowers are greenish white in colour. Petals are 5 in number narrow lancelike, stamens 10 in number. Pods 13-25 cms by 1.8-2.5 cms in size, generally curved, swollen, rigid. Seeds 12-20 glabrous dark reddish brown or black , compressed 8mm long. Flowering season : February - May

Habitat

Bauhinia raemosa grows abundantely in southeastern Asia : India, Pakistan, Srilanka, Cambodia, Myanmar, Thailand, Vietnam. It is usually grown from seeds in evergreen and deciduous forest areas and along the roadside and in villages. Any soil having fair amount of organic matter is suitable for commercial cultivation of this crop.

Plant parts used

The aerial parts of the plant namely the leaf, flower, bark, fruit and bud are used to extract the active phytochemical constituent and thus used for its medicinal purpose.

Phytochemistry

A number of active constituents are found in *Bauhinia racemosa* like alkaloids, coumarins, apigenin, carbohydrates, flavanoids, quercetin, glycosides, protein, rutin, steroids, tannins.

Uses

It has a broad range of pharmacological action such as anti-asthmatic, anthelmentic, antibacterial, antiulcer, anti-malarial, astringent, antimicrobial, blood diseases, internal bleeding,ant-itumour, hepatoprotective ,anti HIV, antioxidant, anti-hyperglycemic.

Excipient Profile

5.2 Tween 80 [16]

Chemical name : Polyoxyethylene 20 sorbitn mono oleate

Molecular formula : C64H124O26

Molecular weight : 1310 g/mol

Structure :

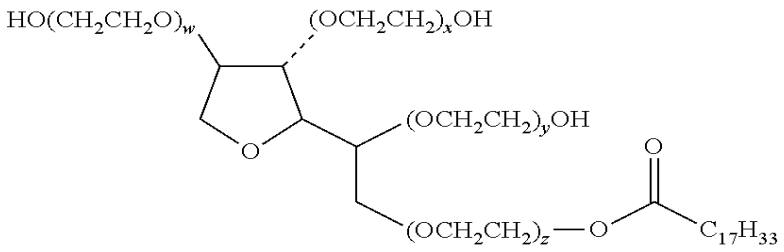


Fig: 11. Structure of Tween 80

Description :

Polysorbate has a characteristic odor and is warm, somewhat bitter in taste. It is soluble in ethanol, water, insoluble in mineral oil and vegetable oil. It should be stored in a well closed container, protected from light, in a cool, dry place.

5.3 Alcohol[16]

Chemical name : ethanol

Molecular formula : C2H6O

Molecular weight : 46.07g/mol

Structure :

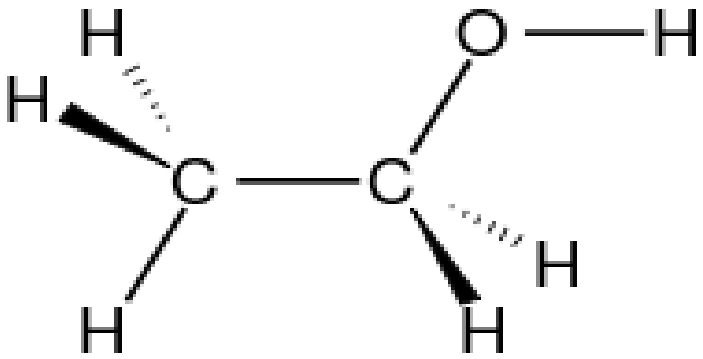


Fig no:12. Structure of ethanol

Description :

Ethanol is produced by the fermentation of sugars by yeasts. It is a volatile, flammable, colorless liquid with a slight chemical odor. It is used as an antiseptic, a fuel, a solvent due to its low freezing point. The molecule is a simple one an ethyl group linked to a hydroxyl group.

5.4 Cinnamon oil [19]

Cinnamomum cassia, called **Chinese cassia** or **Chinese cinnamon**, is an evergreen tree originating in southern China, and widely cultivated there and elsewhere in southern and eastern Asia (India, Indonesia, Laos, Malaysia, Taiwan, Thailand, and Vietnam). It is one of several species of *Cinnamomum* used primarily for their aromatic bark, which is used as a spice. In the United States, Chinese cassia is the most common type of cinnamon used. The buds are also used as a spice, especially in India, and were once used by the ancient Romans.

The tree grows to 10–15 m tall, with greyish bark and hard, elongated leaves that are 10–15 cm long and have a decidedly reddish color when young.

Cinnamomum cassia is a medicinal plant that contains cinnamic aldehyde. Studies of cinnamic aldehyde treatment in mid-aged rats have resulted in alleviation of chronic unexpected stress-induced depressive-like behaviors. Cinnamic aldehyde is an enzyme inhibitor drug, immunologic drug, and an anti-inflammatory drug. It is administered orally to treat behavioral and mental disorders, targeting the hippocampus and the frontal cortex. Current findings might be beneficial in treating subjects in depression.

Due to a blood-thinning component called coumarin, which could damage the liver if taken in huge amounts, European health agencies have warned against consuming high amounts of cassia. Other possible toxins founds in the bark/powder are cinnamaldehyde and styrene.

MATERIALS AND METHODS

MATERIALS

Table No: 2. List of chemicals with name of supplier

S.No	Name of the raw material	Name of the supplier
1	Tween 80	S D fine chemicals ltd, Mumbai
2	Cinnamon oil	Yucca enterprises, Mumbai
3	Sodium dihydrogen orthophosphate	Fine chemical industries, Chennai
4	Di sodium hydrogen orthophosphate	S D fine chemicals ltd, Mumbai
5	Di methyl sulphoxide	Thermo electron LLS pvt ltd

EQUIMENTS

Table No: 3. List of equipments with company name

S.No	Name of the equipments	Name of the supplier
1	Hot air oven	Texcare instruments
2	Sonicator	Vibronics ltd
3	Centrifuge	Remi instruments
4	Zeta potential*	Malvern instruments
5	Weighing balance	Essae - Teraoka ltd
6	Uv- spectrometer	Lab India instruments
7	Particle size analysis*	Malvern instruments
8	Magnetic stirrer	Remi equipments
9	SEM#	Joel JSM,Japan

*Zeta potential and Particle size analysis was obtained from PSG College of Pharmacy, Coimbtaore.

SEM was obatained from Nano science department, Bharatiyar university, Coimbatore.

6.1 Collection and identification of *Bauhinia racemosa*

The plant materials (leaves) were collected from Sulur, Tamilnadu during the month of December 2016.

6.2 Extraction of the leaves of *Bauhinia racemosa* [18,19,20,21,22]

The leaves of *Bauhinia racemosa* were washed with water to remove the mud and other dust particals and then shade dried. These shade dried leaves was then coarsely powdered. Approximately 60 gm of drug powder was extracted with 500 ml ethanol using soxhlet apparatus for 8 hours by hot continuous method. The extract was then concentrated.

6.3 Acute toxicity study of extract of *Bauhinia racemosa* [23,24,25,26,27,28,29,30]

Inbreed wistar albino rats (200 -250 gm) was obtained from the animal house, RVS College of Pharmaceutical Sciences, Sulur. Animals were maintained in a room with controlled temperature (22 ± 2°C) for 12 hour (hr) light / 12 hour dark cycle with free access to food and water. Animal care and research protocols were based on the principles and guidelines adopted by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The acute toxicity study was carried out in both the animals male as well as female wistar albino rats as per Organization Economic Cooperation Development (OECD 423) guidelines. For the evaluation of acute toxicity, the extract 2000mg/kg body weight was used.

The animals were divided into four groups with three animals in each group. Prior to dosing, animals were fasted overnight before being weighed and the dose was administered orally by gavage method.

Control- animals received 1% w/v DMSO.

Group 1 – minimum dose (500mg/ kg body weight suspended in 1% w/v DMSO)

Group 2 – medium dose (1000 mg/ kg body weight suspended in 1% w/v DMSO)

Group 3 – maximum dose (2000 mg/kg body weight suspended in 1% w/v DMSO)

6.3.1 Gross behavior study[31]

The animals were continuously monitored for every 30 min for 3 hours (hr), 4th hr and 24th hr for gross behavioral changes and mortality. Once daily cage side observations included changes in skin, fur, eyes, autonomic nervous system (salivation, lacrimation and defecation) and central nervous system (ataxia, tremors and coma) changes. Mortality , if any was determined over a period of 14 days also recorded.

6.3.2 Body weight analysis[32]

Individual body weight of animal was recorded before the administration of a drug on 0 day of the study and the 14th day of the experiment before withdrawal of the blood from the individual animals. Changes in the body weight of the treated animals were compared with the control animals.

6.3.3 Hematological study[33]

Blood samples were collected by retro orbital puncture of all the treated animals and control animals. samples were analyzed for routine hematological parameters like hemoglobin, white blood corpuscle, red blood corpuscle, packed cell volume and platelet counts. Blood cell counts were done with blood smears. The above mentioned hematological parameters were analyzed.

6.3.4 Biochemical study[32]

For the determination of Serum Glutamic Oxaloacetate Transminase (SGOT), Serum Glutamic Pyruvate Transaminase (SGPT), total cholesterol, triglycerides, sugar, protein and creatinine, blood samples were collected separately for each of the group (control and treated) and then analyzed .

6.3.5 Gross necropsy[31] and histopathological studies[34]

After administration of drug at the end of 14th day, the animals were sacrificed by cervical dislocation method. All animals in the study were subjected to a full detailed gross necropsy like careful examination of the external surface of the body and abdominal cavities and their contents are examined.

The organs like liver, heart and kidney were dissected and washed with cold saline. All the tissue samples were fixed in 10% volume/volume (v/v) formalin and stained with hematoxylin and eosin stain for histopathological examination. All the tissue samples were examined under light microscopy.

6.4 Determination of the thrombolytic activity of the extract[35,36,37,38,39,40,41,42]

The dry crude extract (1mg) was suspended in 1ml of DMSO. 1ml/tube of blood was added into pre-weighed sterile micro centrifuge tube and incubated at 37°C for 45 mins. After clot formation, the serum was completely removed without disturbing the clot and each tube having the clot was again weighed to determine the clot weight.

Clot weight = weight of clot containing tube – weight of tube alone

To each micro centrifuge tube containing the pre weighed clot, 100µl DMSO solutions of different partitionates along with the crude extract was added separately. As, a positive control 100µl of streptokinase (SK) and as a negative non thrombolytic control 100µl of distilled water and 100µl of DMSO were added separately to the different control tubes. All the tubes are then incubated at 37° C for 90 mins and observed for clot lysis. After incubation the released fluid was removed and the tubes were weighed again. The difference in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown.

% of clot lysis = (weight of released clot/ clot weight)* 100.

6.5 Compatibility studies of the extract [43]

6.5.1 Components screening by solubility determination

One of the important factors to formulate a stable nanoemulsion system is choosing components of nanoemulsion which are oil, surfactants and co-surfactants. The components were selected by determining the solubility of the extract in different oils like coconut oil, neem oil, black cumin seed oil, cinnamon oil, soyabean oil; surfactants like tween 80, tween 20, span 80; co-surfactants like acetone, ethanol, methanol.

1mg of the extract was dissolved in 1ml of the above mentioned exipients and the solubility was determined by visual observation with eyes.

6.5.2 Drug compatibility studies

The physical stability of the excipient with drug extract has to be determined. The drug extract is mixed with the components used in the formulation in 1:1 ratio. These sample mixture is then placed in two closed containers each at room temperature 25±1°C and accelerated temperatures 40±1°C for 8 weeks. The mixtures were then observed for any specific changes by visual observations.

6.6 Preparation of nanoemulsion of the extract [44,45,46,47,48,49,50,51,52,53,54]

Nanoemulsion was prepared by the ultrasonic emulsification method. Briefly, accurately weighed quantity of drug was dissolved in measured quantity of ethanol (co-surfactant). This was followed with the addition of measured amount of Tween 80 (surfactant). This mixture was called as Smix. By varying the concentration of surfactant and the co- surfactant various combinations of Smix ratios were prepared. The mixture was allowed to get homogenized properly by the help of an magnetic stirrer. To this cinnamon oil was added followed by required amount of distilled water and allowed to get a uniform, homogenized emulsion. This formed emulsion was then sonicated for 30 mins to get the nano emulsion. By, varying the Smix ratio as 2:1, 3:1, 4:1and the concentration of oil and water 9 batches of nanoemulsion were formulated.

Table no: 4. Formulation batches

Sl.no	Ingredients	Formulation code								
		F1	F2	F3	F4	F5	F6	F7	F8	F9
1	Drug extract*	100	100	100	100	100	100	100	100	100
2	Tween 80	40	40	40	40	52.5	52.5	52.5	64	64
3	Ethanol	20	20	20	20	17.5	17.5	17.5	16	16
4	Cinnamon oil	20	15	10	5	15	10	5	10	5
5	Distilled water	20	25	30	35	15	20	25	10	15

* Marked values are in milligrams. All other values are in ml.

6.7Characterization of prepared nanoemulsion

6.7.1 Particle size analysis [49]

For the determination of particle size optical microscopy method was employed. The particle size distribution studies of the nanoemulsion was carried out using particle size analyzer (Particle size analyzer Hydro 2000 SM, Malvern Instruments, UK).

10 ml of the sample is dispersed in 120 ml of dispersion medium and analysis was performed at a scattering angle of 90° and at a temperature of 25°C for particle size analyzer. For the measurement of particle size by zeta seizer, each sample (1-2 ml) was placed in a disposable zeta cell and run to obtain the zeta size value at 25°C. The analysis was performed thrice and the average value was reported.

6.7.2 Zeta potential determination [50]

Zeta potential values of nanoemulsion was determined by using zeta sizer version 6.2 (Malvern Instruments, UK). The nanoemulsion (1 ml) was dispersed in a disposable zeta cell. Palladium electrode was inserted in distilled water in the cuvette and the analysis was performed at 25°C. All the measurements were done in triplicate and average value was reported , which measures the distribution of the electrophoretic mobility of particles using the laser Doppler velocity technique. The analyzer calculated the zeta potential from the measured velocity using the smoluchowski equation.

6.7.3 Particle morphology study [49]

2ml of nanoemulsion was placed on stub and dried for overnight under room temperature. Prior to loading the samples for taking the photomicrograph, samples are coated with a an electron dense coating materials like gold, palladium or a combination of both, to enhance the signal emitted by the sample by providing heavy metal atoms with incident beam of an electronand to conduct the accumulated sample charge and heat to the sample holder. The coating process was either carried out using sputter coating or thermal vacuum evaporation.

6.7.4 In vitro release study [49]

In vitro diffusion studies was performed for all the formulated batches using a dialysis technique. The dialyzing medium was phosphate buffer ph 7.4. One end of pretreated cellulose dialysis tubing (7 cm in length) was tied with thread and then 1 ml of self nano emulsifying formulation was placed in it along with 0.5 ml of dialyzing medium. The other end of the tubing was also securecd with thread and was allowed to rotate freely in 200 ml of dialyzing medium and stirred continuously at 100 rpm with magnetic bead on magnetic plate 37°C. Aliquots of 1 ml were removed at different time intervals and diluted further with the buffer.

Volume of aliquots was replaced with fresh dialyzing medium each time. These samples were analyzed quantitatively for drug dialyzed across the membrane at corresponding time by using UV- visible spectrophotometer.

6.8 Stability study of nanoemulsion [52]

The stability of prepared nanoemulsion was tested by subjecting them to different stress conditions of mechanical and accelerated temperatures. The prepared formulations were evaluated for the following attributes:

6.8.1 Mechanical stress study

The different formulations were centrifuged at 2000 rpm for different intervals and noted the volume of phase separation of nanoemulsion.

6.8.2 Accelerated temperature study

The phase separation study was performed for the evaluation of prepared formulations by storing them at different accelerated temperatures. Three batches of the same formulations were stored in sealed and scaled containers at room temperature (25 ± 1°C), at refrigerated temperature (4 ± 1°C) and at higher temperature (40 ± 1°C) for 30 days. After 1, 10, 20 and 30 days each formulation was evaluated by visual observation for phase separation

RESULTS AND DISCUSSION

7.1 Collection and identification of *Bauhinia racemosa*

The plant materials (leaves) were collected from Sulur, Tamilnadu during the month of march 2016. It was authenticated by Dr. M. Palanisamy, Scientist 'D', In charge Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamilnadu – 641003. Register No: BSI/SRC/5/23/2016/Tech./585.

7.2 Extraction of the leaves of *Bauhinia racemosa*

Extraction of the leaves of *Bauhinia racemosa* was carried out as per the procedure given under materials and methods section 6.2.

The dried leaves powder approximately 60 gm was extracted with 500 ml of ethanol for 8 hours (hrs) by hot continuous extraction method by using soxhlet apparatus. The extract was concentrated. The yield was found to be 15.95% w/v.

7.3 Acute toxicity study of extract of *Bauhinia racemosa*

Acute toxicity study was carried out as per the procedure described in materials and methods section 6.3.

During the study period of 14 days, there was no mortality or morbidity observed in the experimental animals followed by single oral administration of extract.

7.3.1 Gross behavior study

Gross behavior study was carried out as per the procedure described in materials and methods section 6.3.1

The results of the study revealed no adverse change in cage side observation in animals. The results are shown in the Table no: 5, 6 and 7. All the animals in the control and treated groups were found healthy as well as active.

Table no: 5. Gross behavior study of treated animals. (Group - 1. Minimum dose)

Observation	0.5 hr	1.0 hr	1.5 hr	2.0hr	2.5 hr	3.0 hr	4.0 hr	24 hr
Skin and Fur	+	+	+	+	+	+	+	+
Ear	+	+	+	+	+	+	+	+
Eyes	+	+	+	+	+	+	+	+
Salivation	+	+	+	+	+	+	+	+
Diarrhoea	–	–	–	–	–	–	–	–
Lacrimation	–	–	–	–	–	–	–	–
Ataxia	–	–	–	–	–	–	–	–
Tremors	–	–	–	–	–	–	–	–
Coma	–	–	–	–	–	–	–	–
Mortality	–	–	–	–	–	–	–	–

Table no: 6. Gross behavior study of treated animals. (Group - 2. Medium dose)

Observation	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	3.0 hr	4.0 hr	24 hr
Skin and fur	+	+	+	+	+	+	+	+
Ear	+	+	+	+	+	+	+	+
Eyes	+	+	+	+	+	+	+	+
Salivation	+	+	+	+	+	+	+	+
Diarrhea	–	–	–	–	–	–	–	–
Lacrimation	–	–	–	–	–	–	–	–
Ataxia	–	–	–	–	–	–	–	–
Tremors	–	–	–	–	–	–	–	–
Coma	–	–	–	–	–	–	–	–
Mortality	–	–	–	–	–	–	–	–

Table no: 7. Gross behavior study of treated animals. (Group – 3. Maximum dose)

Observation	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	3.0 hr	4.0 hr	24 hr
Skin and Fur	+	+	+	+	+	+	+	+
Ear	+	+	+	+	+	+	+	+
Eyes	+	+	+	+	+	+	+	+
Salivation	+	+	+	+	+	+	+	+
Diarrhea	–	–	–	–	–	–	–	–
Lacrimation	–	–	–	–	–	–	–	–
Ataxia	–	–	–	–	–	–	–	–
Tremors	–	–	–	–	–	–	–	–
Coma	–	–	–	–	–	–	–	–
Mortality	–	–	–	–	–	–	–	–

[+ Normal - Absence]

The control animals were also observed and there was no change in the above parameters.

7.3.2 Body weight analysis

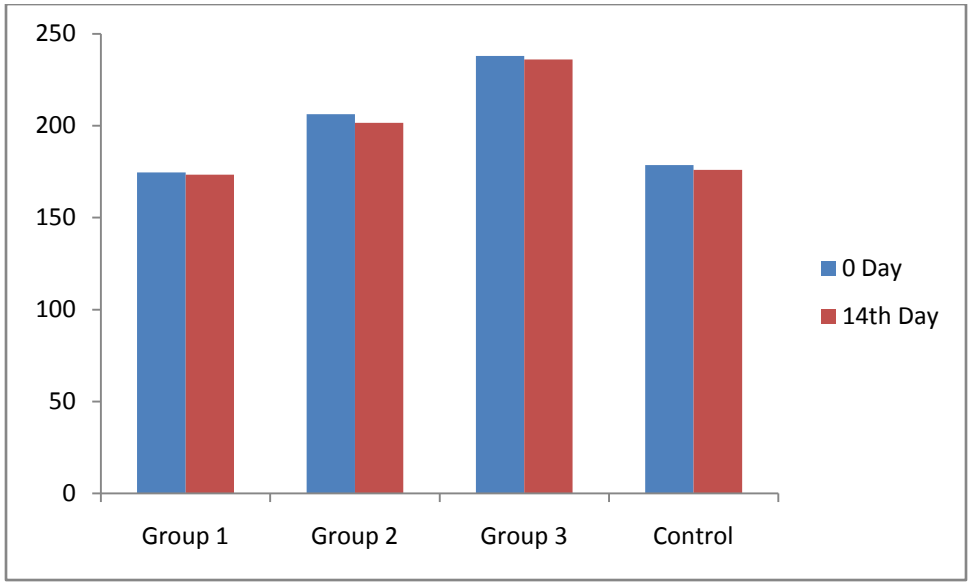
Body weight analysis was carried out as per the procedure described in materials and methods section 6.3.2.

No considerable changes were observed in the body weight between the groups and the data are tabulated in Table no: 8.

Table no : 8. Effect of leaf extract on body weight.

Groups	Body weight (gm) on day 0 (n=3)	Body weight (gm) on day 14(n=3)
Control	178.66 ± 6.02	176.00 ± 3.60
Group – 1 (minimum dose)	174.66 ± 4.51	173.33 ± 1.53
Group – 2 (medium dose)	206.33 ± 6.02	201.66 ± 8.08
Group – 3(maximum dose)	238.00 ± 7.54	236.00 ± 3.60

Fig no: 14. Effect of leaf extract on body weight.



7.3.3 Hematological study

Determination of the hematological parameters were carried out as per the procedure described in materials and methods section 6.3.3.

All hematological parameters of test group was compared to that of the control group. There were no significant changes in the tested hematological parameters like

- Hemoglobin (Hb)
- White Blood Corpuscle (WBC)
- Red Blood Corpuscle (RBC)
- Packed Cell Volume (PCV)
- Platelet counts.

The results are shown in Table no: 9.

Table no: 9. Effect of leaf extract on hematological parameters

Parameters	**Control group (n=3)**	**Group – 1 (minimum dose). (n=3)**	**Group -2 (medium dose). (n=3)**	**Group-3 (maximum dose). (n=3)**
Hb (g %)	13.76 ± 0.44	13.36 ± 0.61	13.03 ± 03	14.23 ± 0.30
WBC (10^3/ cubic millimeter)	12.3 ± 0.4	13.2 ± 0.3	11.7 ± 0.65	12.23 ± 0.45
RBC (10^6/cu mm)	5.71 ± 0.07	5.44 ± 0.187	5.37 ± 0.40	5.73 ± 0.04
PCV (%)	43.8 ± 1.80	42.26 ± 0.40	40.63 ± 0.84	43.56 ± 0.90
Platelets (10^3/ cu mm)	852.33 ± 11.67	703.33 ± 12.58	817.33 ± 12.74	895 ± 9.16

[The results are expressed as mean ± S.D]

Fig no:15. Effect of leaf extract on hematological parameters. (Hb, WBC, RBC).

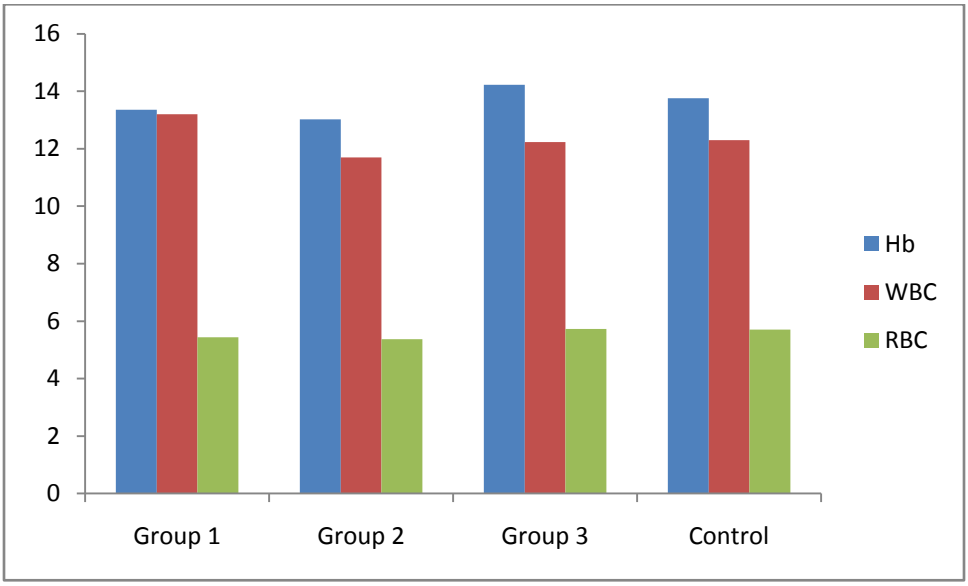
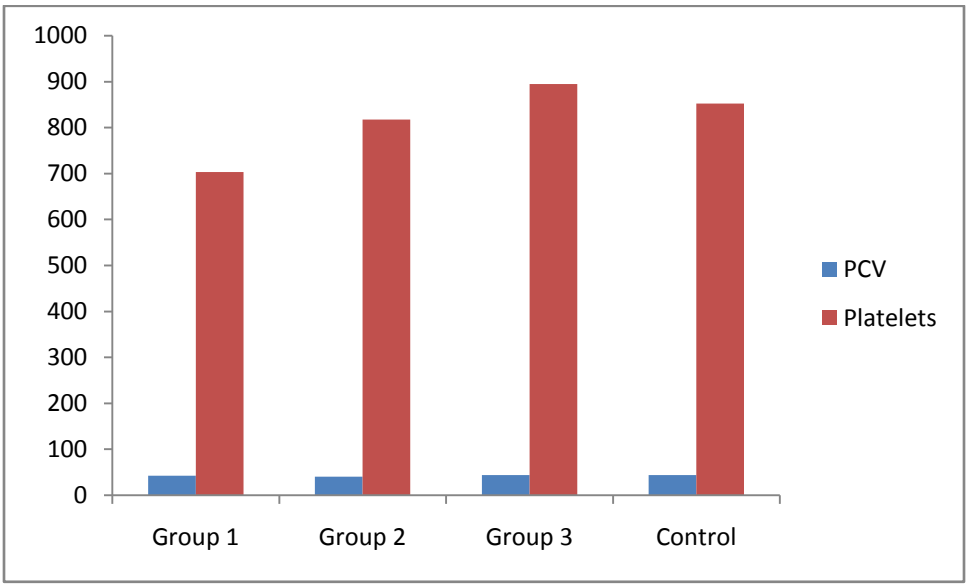


Fig no: 16. Effect of leaf extract on hematological parameters. (PCV, Platelets).



7.3.4 Biochemical study

Determination of biochemical parameters was carried out as per the procedure described in materials and methods section 6.3.4.Evaluation of hepatic and renal function is of prime importance to assess the inherent toxic properties of drugs. However the results of assay of these enzymes revealed no difference between the control and treated group. There were no significant changes in the treated group when compared to the control group. The bio-chemical parameters of the control and treated group are presented in Table no: 10.

- Serum Glutamic Oxaloacetic Transaminase (SGOT)
- Serum Glutamic Pyruvic Transaminase (SGPT)
- Total cholesterol
- Triglyeride
- Protein
- Creatinine

Table no: 10. Effect of leaf extract on bio-chemical parameters.

Parameters	**Control group. (n=3)**	**Group – 1 (minium dose). (n= 3)**	**Group – 2 (medium dose). (n=3)**	**Group – 3 (maximum dose). (n=3)**
SGPT (IU/L) (International unit/ liter)	103.4 ± 0.45	103.56 ± 1.76	104.73 ± 1.78	102.33 ± 0.47
SGOT (IU/L)	277.3 ± 0.6	272.3 ± 2.55	279.73 ± 4.65	270.23 ± 0.7
Total cholesterol (mg/dl)	67.66 ± 0.54	60.63 ± 1.52	73.46 ± 0.90	66.23 ± 0.41
Triglycerides (mg/dl)	81.16 ± 0.44	79.86 ± 1.07	83.4 ±0.55	79.13 ± 0.60
Protein (mg/dl)	7.46 ± 0.15	7.26 ± 0.40	7.2 ± 0.35	7.13 ± 0.2
Creatinine (mg/dl)	0.68 ± 0.03	0.62 ± 0.02	0.64± 0.03	0.66 ± 0.03

[The results are expressed as mean ± S.D]

Fig no: 17. Effect of leaf extract on bio-chemical parameters.(SGPT,SGOT,Cholesterol.

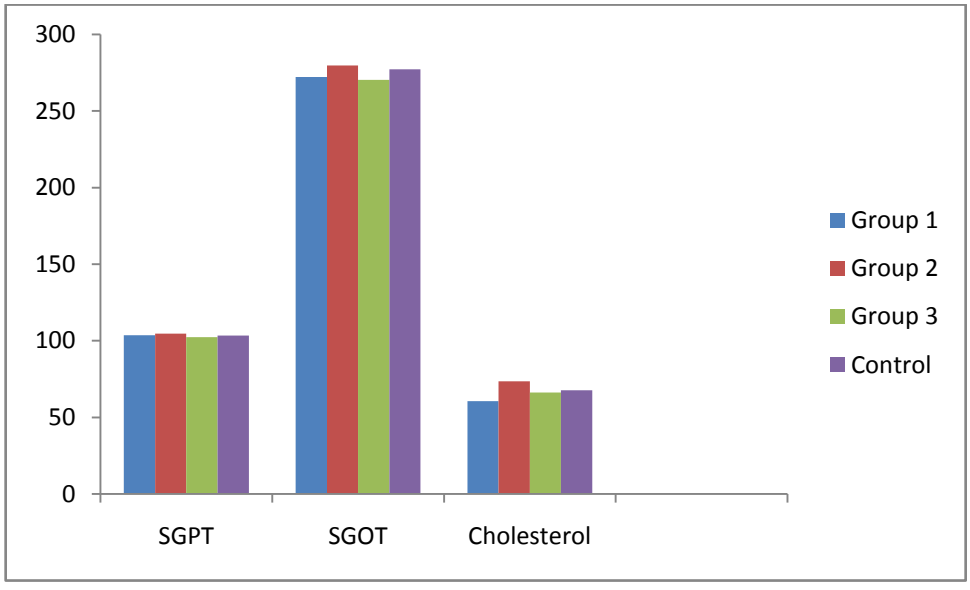
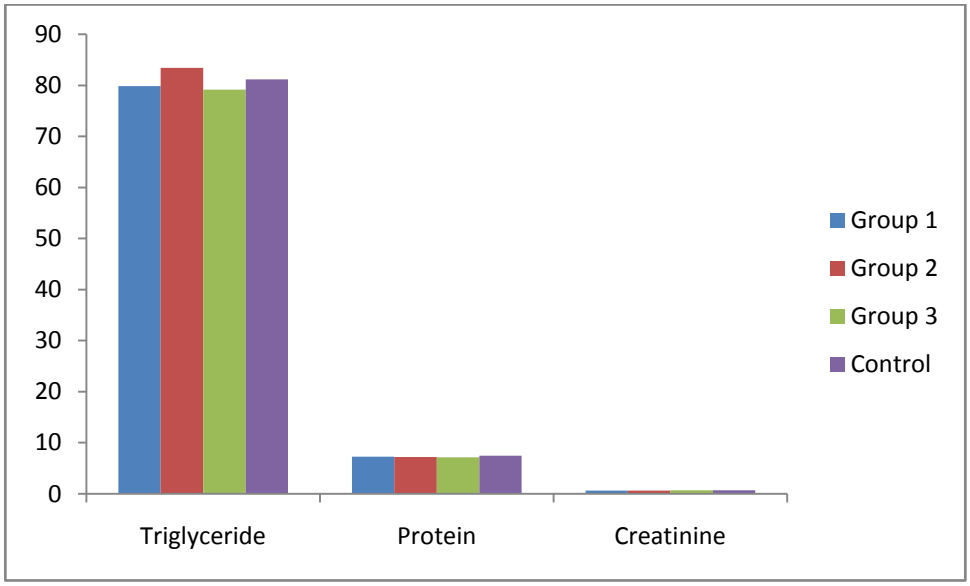


Fig no: 18. Effect of leaf extract on bio-chemical parameters. (Triglyceride, Protein, Creatinine).



7.3.5 Gross necropsy and histopathological studies

Gross necropsy study was carried out as per the procedure described in materials and methods section 6.3.5.

Morphological observation of abdominal cavities, their content, organs like liver, heart and kidney were examined and found that there was no sign of inflammation or toxicity in all the groups. The results are summarized as follows.

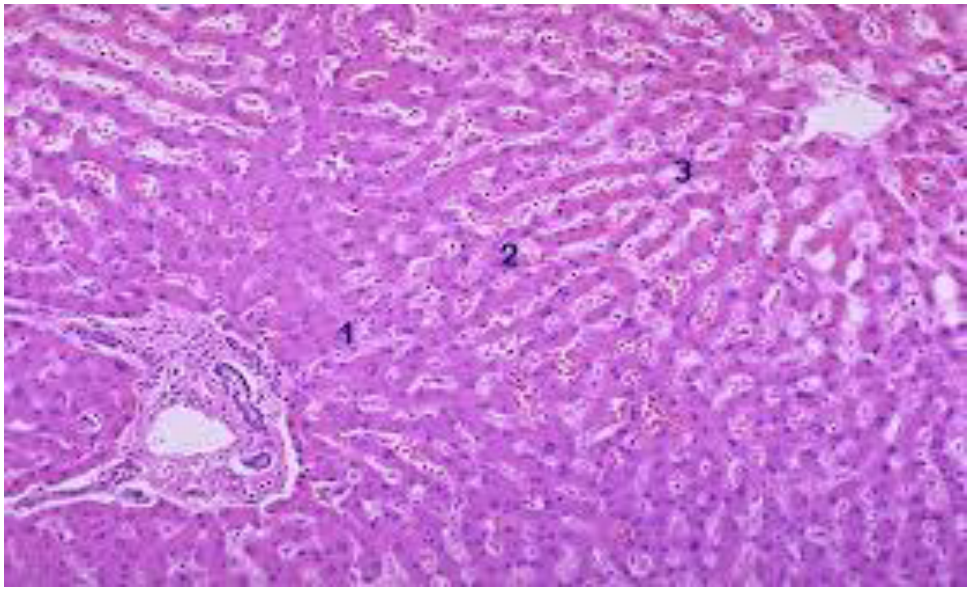


Fig no: 19. Histopathological slide of Control group liver.

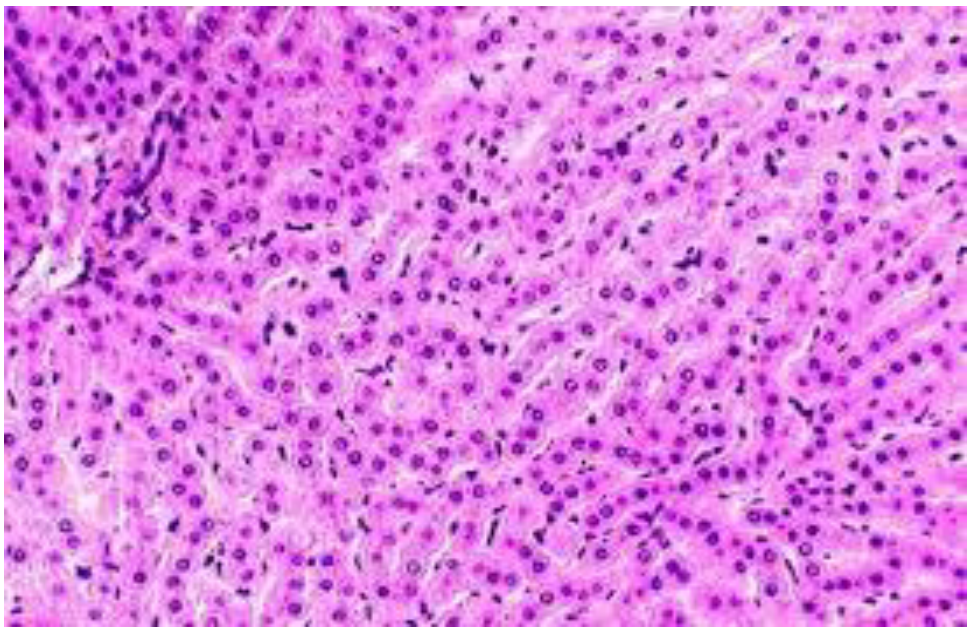


Fig no: 20. Histopathological slide of treated (group – 1) liver.

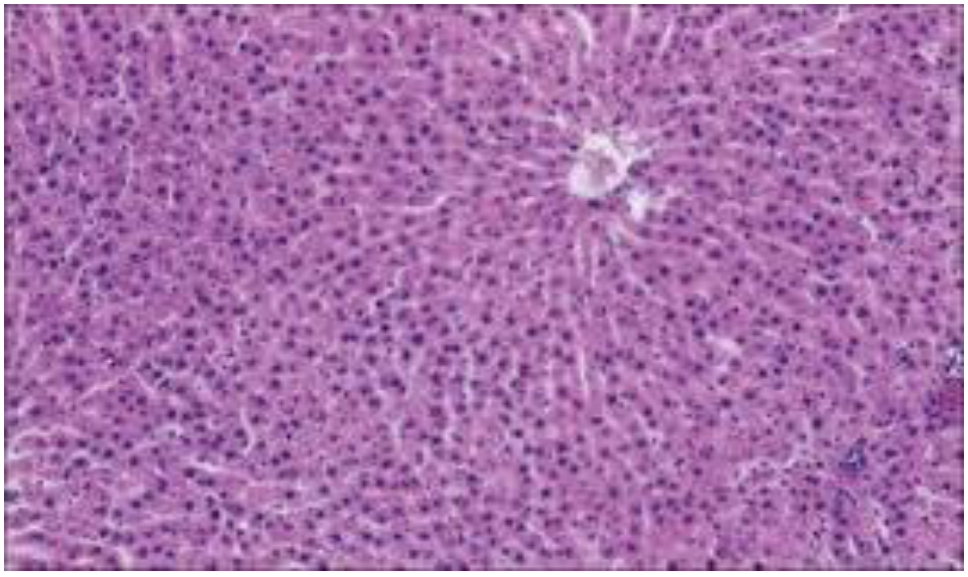


Fig no: 21. Histopathological slide of treated (group -2) liver.

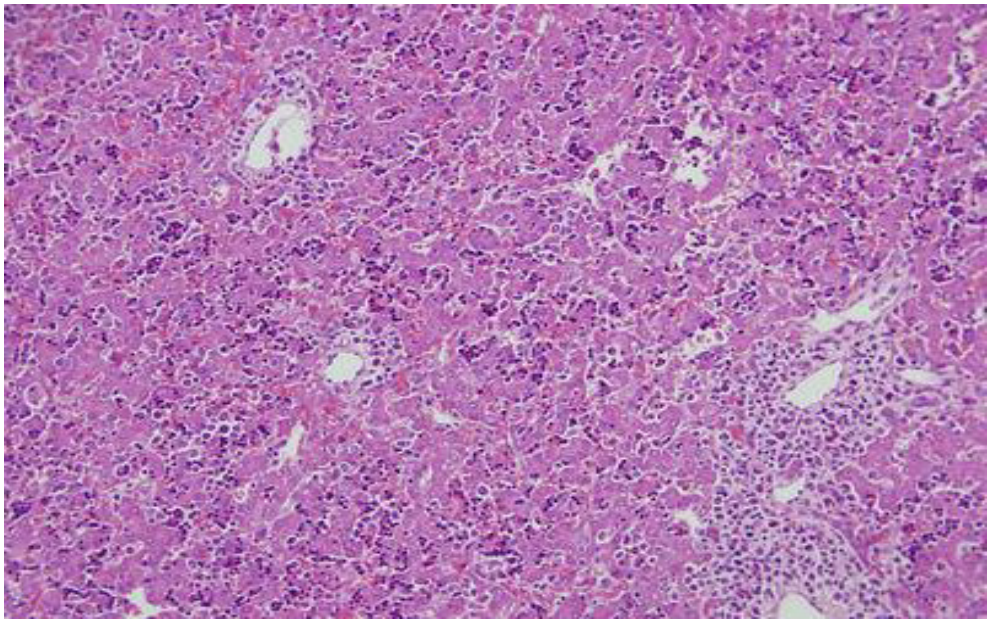


Fig no: 22. Histopathological slide of treated (group – 3) liver.

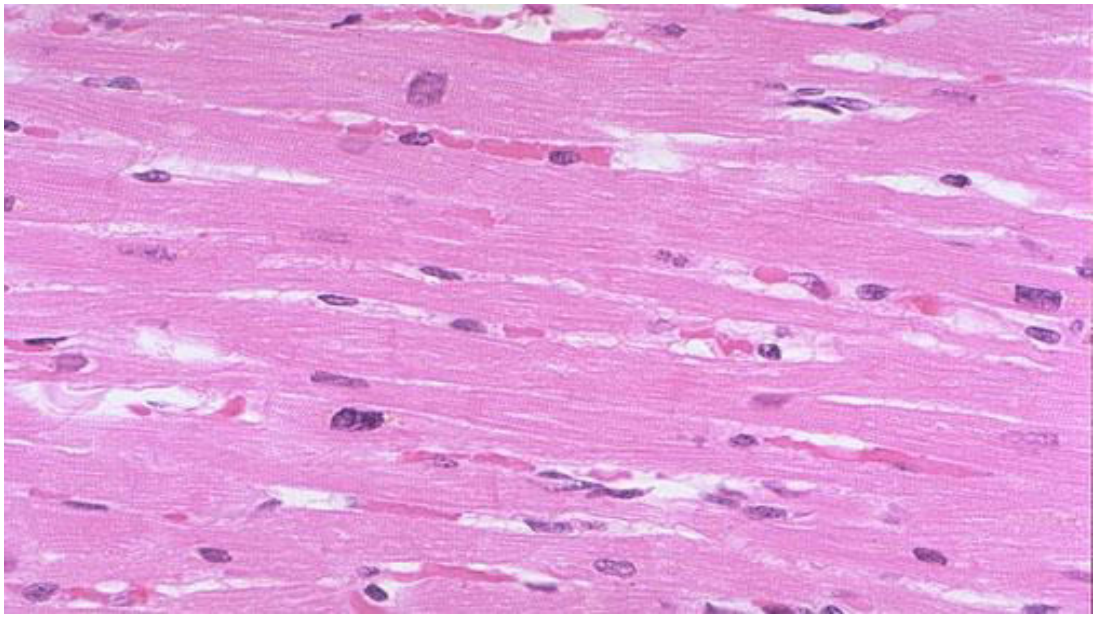


Fig no: 23. Histopathological slide of control group heart.

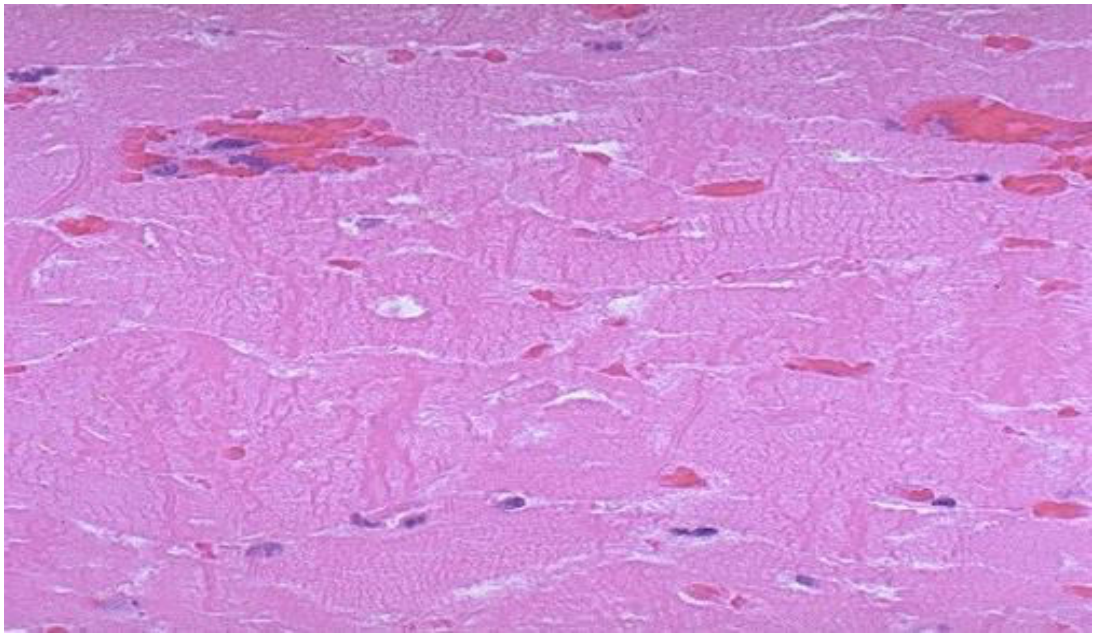


Fig no: 24. Histopathological slide of treated (group – 1) heart.

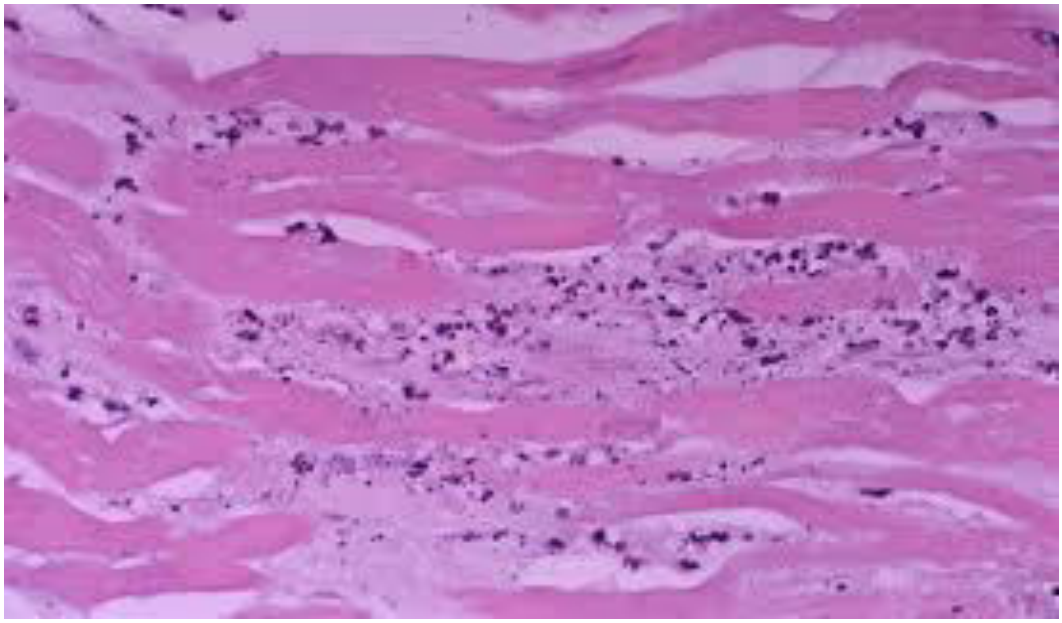


Fig no: 25. Histopathological slide of treated (group – 2) heart.

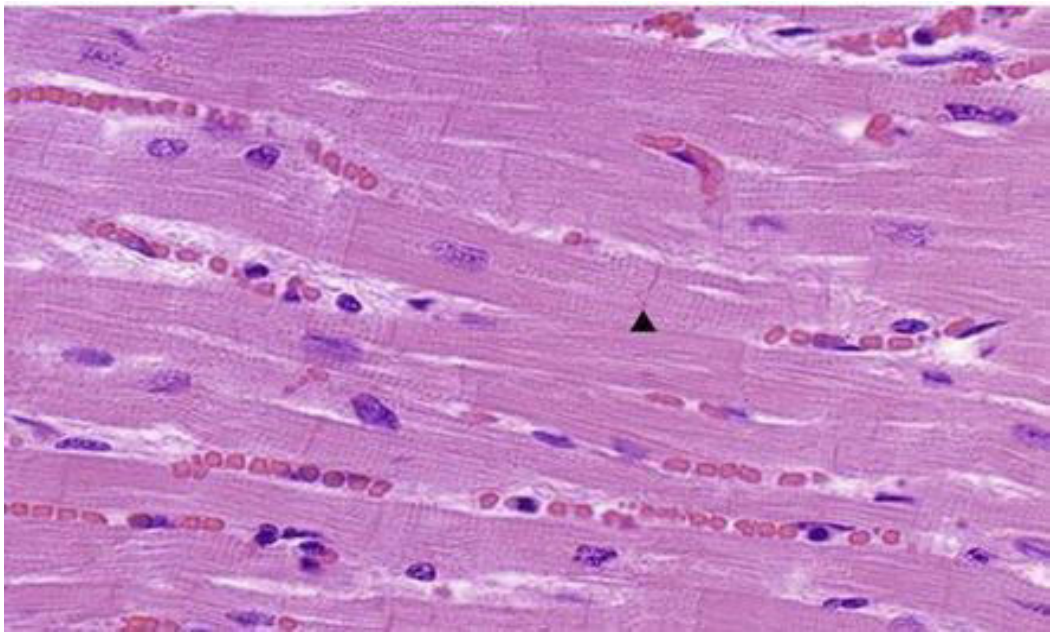


Fig no: 26. Histopathological slide of treated (group- 3) heart.

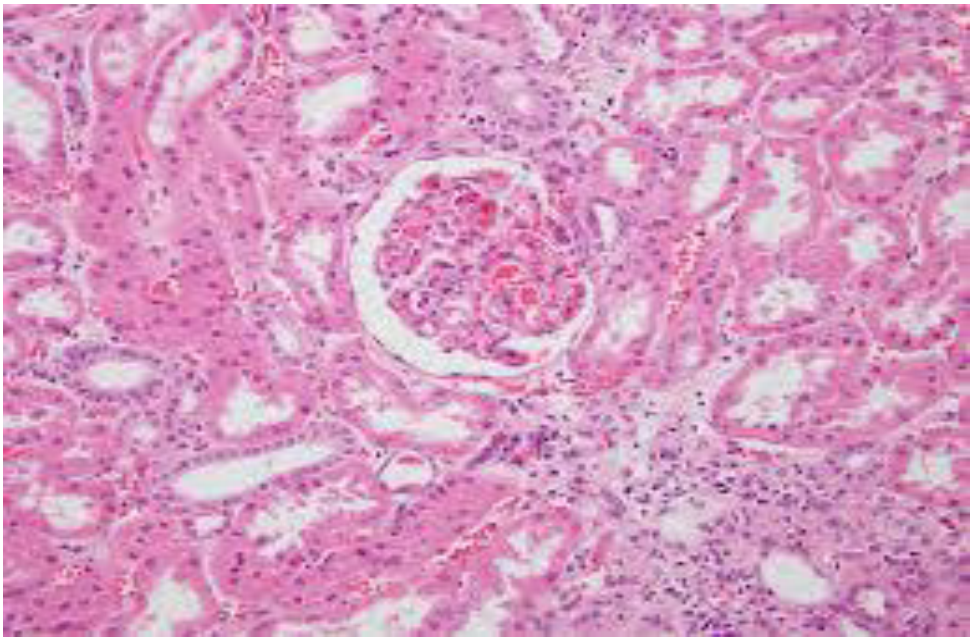


Fig no: 27. Histopathological slide of control kidney.

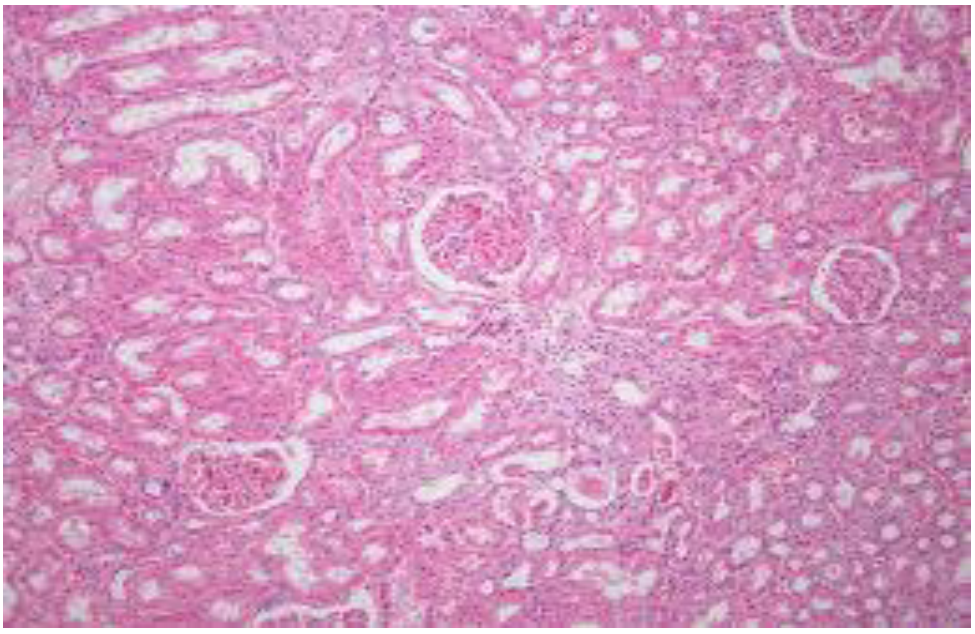


Fig no: 28. Histopathological slide of treated (group – 1) kidney.

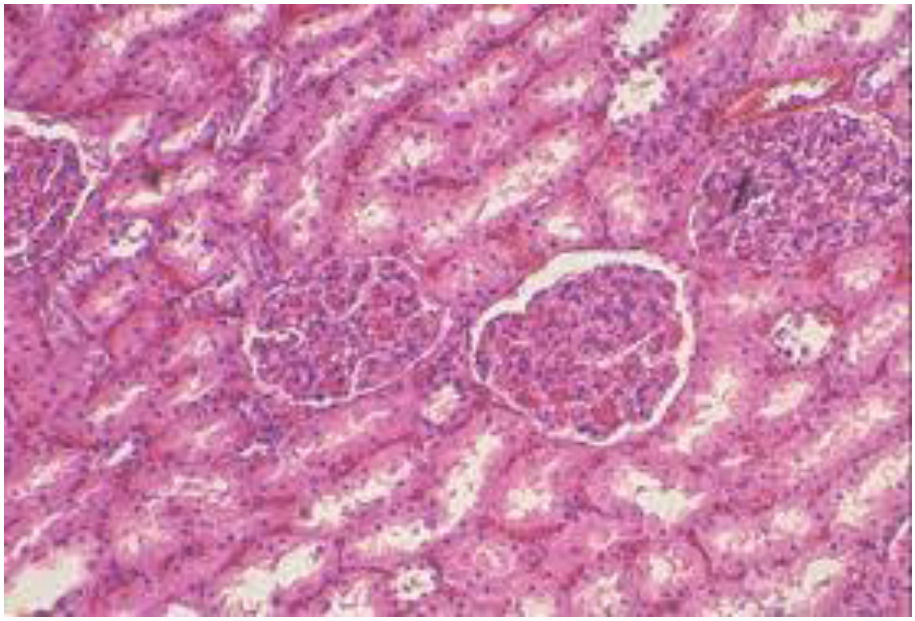


Fig no: 29. Histopathological slide of treated (group – 2) kidney.

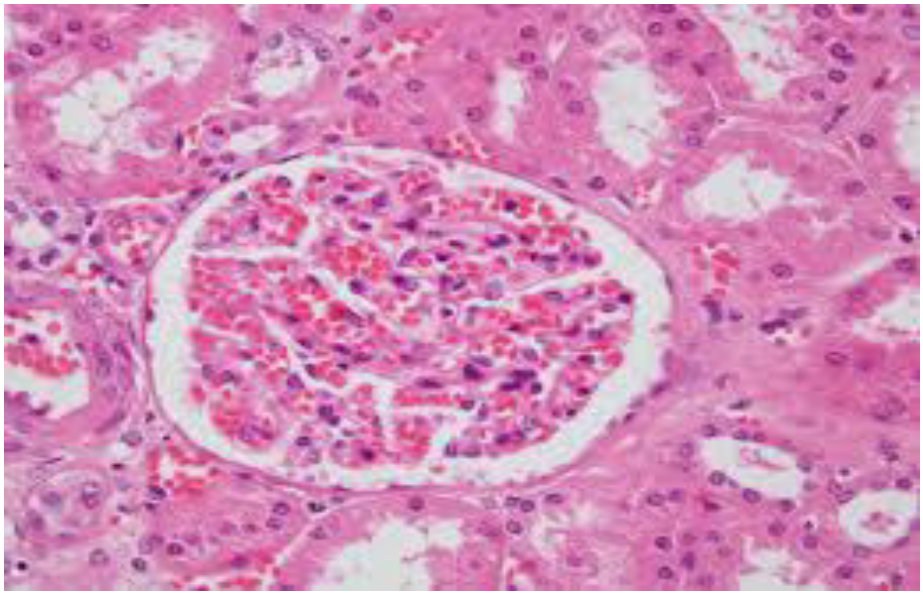


Fig no: 30. Histopathological slide of treated (group - 3) kidney.

Histopathological study was carried out as per the procedure described in materials and methos section 6.3.5.

The histopathological investigation of the liver tissue for the control group and treated group were examined. The corresponding results are presented in figures 19- 22. Control liver tissue pointed out the presence of normal hepatocytes and shown a mild fatty degeneration. The control liver cellular architecture was almost retained in treated animals. Hence, it did not show any toxicity in the liver.

The histology of the heart of both the control and treated were shown normal myocardial fiber and no significant pathology (shown in figures 23-26). The cellular architecture of the kidney of the control and treated groups studied by the histopathological analysis was presented in figures 27-30. The kidney of the control group showed normal glomeruli and tubules. The histopathology of treated kidney when compared with control showed no significant pathology.

The results of acute toxicity studies clearly demonstrate that the extract treated animals are devoid of any toxic sign and indicates that it is safe at the dose of 2000 mg/kg body weight of animal.

7.4 Determination of the thrombolytic activity of the extract

Determination of thrombolytic activity was carried out as per the procedure given under materials and methods section 5.4.The results obtained were significant. The results are given in the Table no: 11.

Table no: 11. Effect of leaf extract/ drug on *in-vitro* clot lysis

Extract/Drug	**Percentage of clot lysis (Mean ± S.D)**
Streptokinase	91.13 ± 1.29
Water	2.725 ± 0.98
Ethanolic extract of *B.racemosa* leaves	90.77 ± 1.67
DMSO	1.130 ± 0.25

7.5 Compatibility studies of the extract

7.5.1 Components screening by solubility determination

The components of formulation were screened for solubility based on the procedure given under materials and methods section 6.5.1.

The results are as given in the Table no: 12. Acetone was not selected because of its volatile nature and methanol was not selected because of its toxic nature.

Table no: 12. Screening of components for solubility.

S.NO	COMPONENTS	SOLUBILITY
1	OIL: Coconut oil Neem oil Black cumin seed oil Cinnamon oil Soyabean oil	 Sparingly soluble Insoluble Sparingly soluble Completely soluble Sparingly soluble
2	SURFACTANT: Tween 80 Tween 20 Span80	 Sparingly soluble Insoluble Insoluble
3	CO-SURFACTANT: Ethanol Acetone methanol	 Completely soluble Soluble Soluble

7.5.2 Drug compatibility studies

The drug compatibility studies were carried out as per the procedure given under materials and methods section 6.5.2.The mixtures were observed for any specific changes. The observations are as given below in Table no:13.

Table no: 13. Drug compatibility study

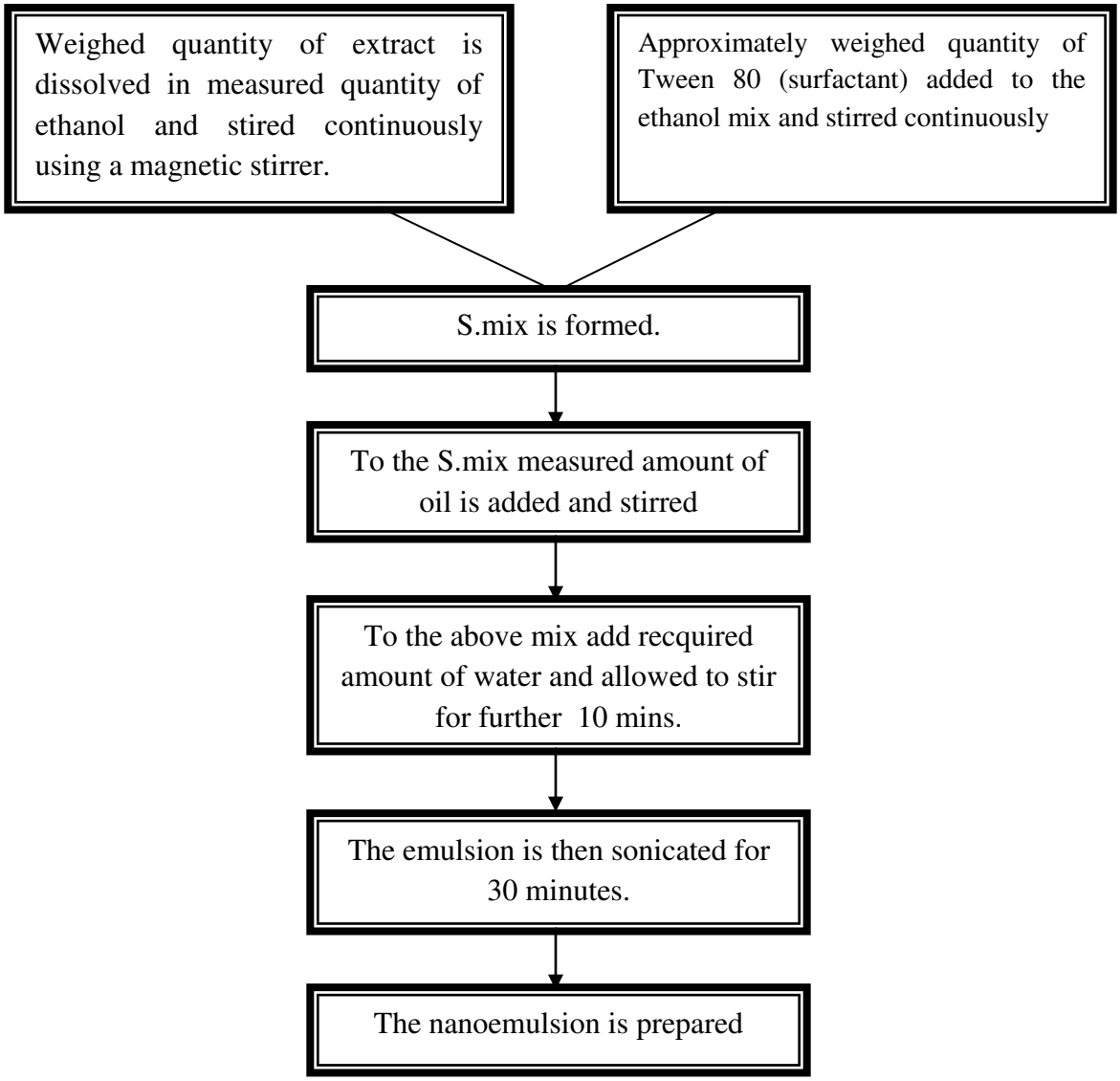
MIX-TURE	25±1°C								40±1°C							
	1 Wk	2 Wk	3 Wk	4 Wk	5 Wk	6 Wk	7 Wk	8 Wk	1 Wk	2 Wk	3 Wk	4 Wk	5 Wk	6 Wk	7 Wk	8 Wk
EXTRACT+CINNAMON OIL	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC
EXTRACT+ETHANOL	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC
EXTRACT+TWEEN 80	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC	NSC

NSC – No Specific Change. **Wk – Week.**

7.6 Preparation of nanoemulsion of the extract

The nanoemulsion was prepared as per the procedure given under materials and methods section 6.6. The step wise procedure for the preparation of nanoemulsion is given in the schematic diagram as follows.

Fig no: 31. Schematic representation of preparation steps.



7.7 Characterization of prepared nanoemulsion

7.7.1 Particle size analysis

Particle size analysis was carried out as per the procedure given under materials and methods section 6.7.1.

The particle size and size distribution are important for nanoparticles drug delivery. The size distribution was given by the polydispersibility index . The lower the value is the narrow the size distribution or the more uniform of the nanoparticles.

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	175.5	Peak 1:	194.4	100.0	65.22
PdI:	0.116	Peak 2:	0.000	0.0	0.000
Intercept:	0.923	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

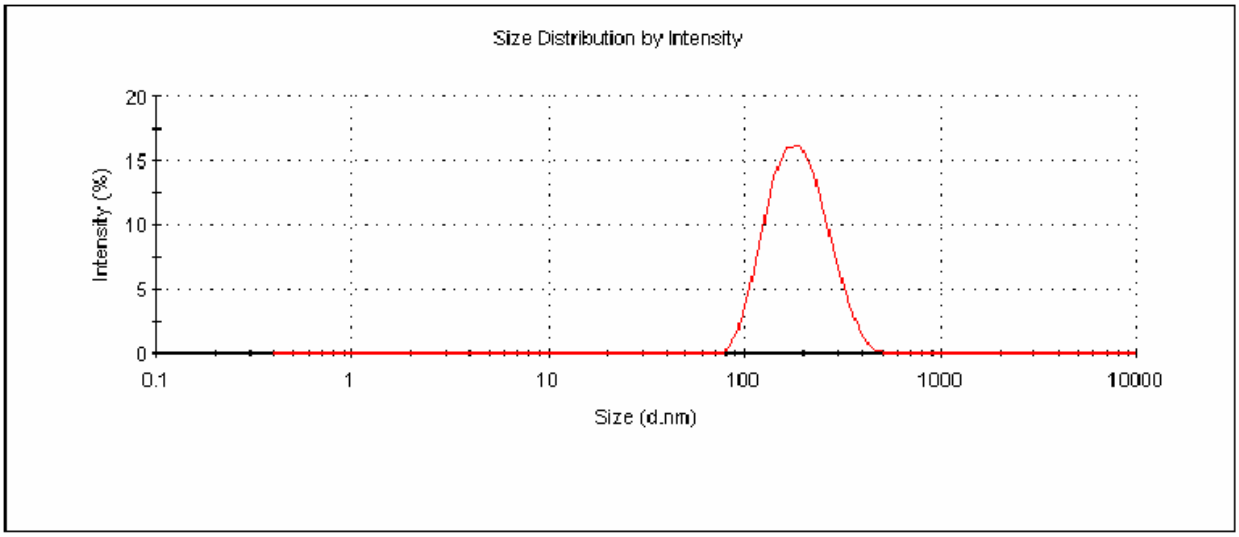


Fig no: 32. Particle size distribution of the prepared nanoemulsion.

7.7.2 Zeta potential determination

Zeta potential determination was carried out as per the procedure described in materials and methods section 6.7.2.

The zeta potential represents an index for particle stability. This stability is important in preventing aggregation of particles. The zeta potential of the optimized formulation was found to be milli volt (mV). Higher the zeta potential maximum is the stability of nano particles.

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -3.62	Peak 1:	-3.62	100.0	3.43
Zeta Deviation (mV): 3.43	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.160	Peak 3:	0.00	0.0	0.00

Result quality : Good

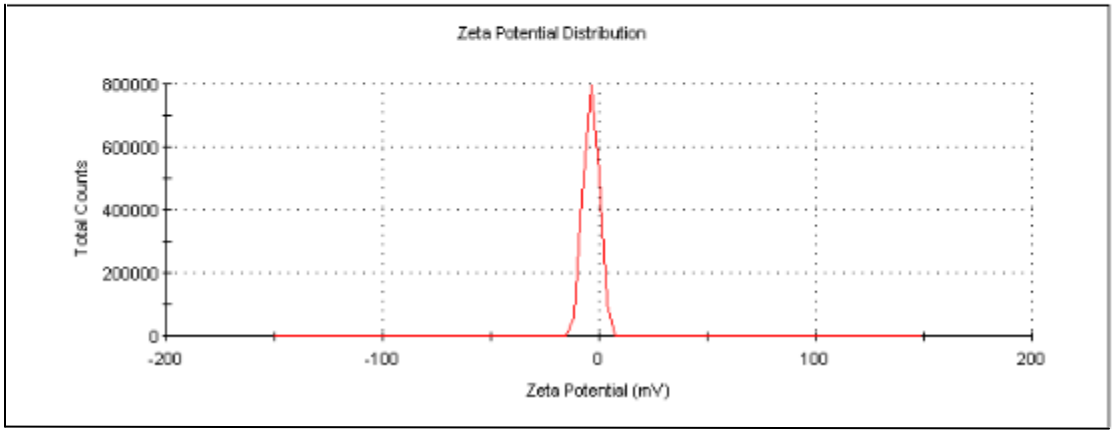


Fig no: 33. Zetapotential of the prepared nanoemulsion.

7.7.3 Particle morphology study

Particle morphology study was carried out as per the procedure described in materials and methods section 6.7.3.

The prepared nanoemulsion are subjected to morphological analysis by Scanning Electron Microscopy (SEM)

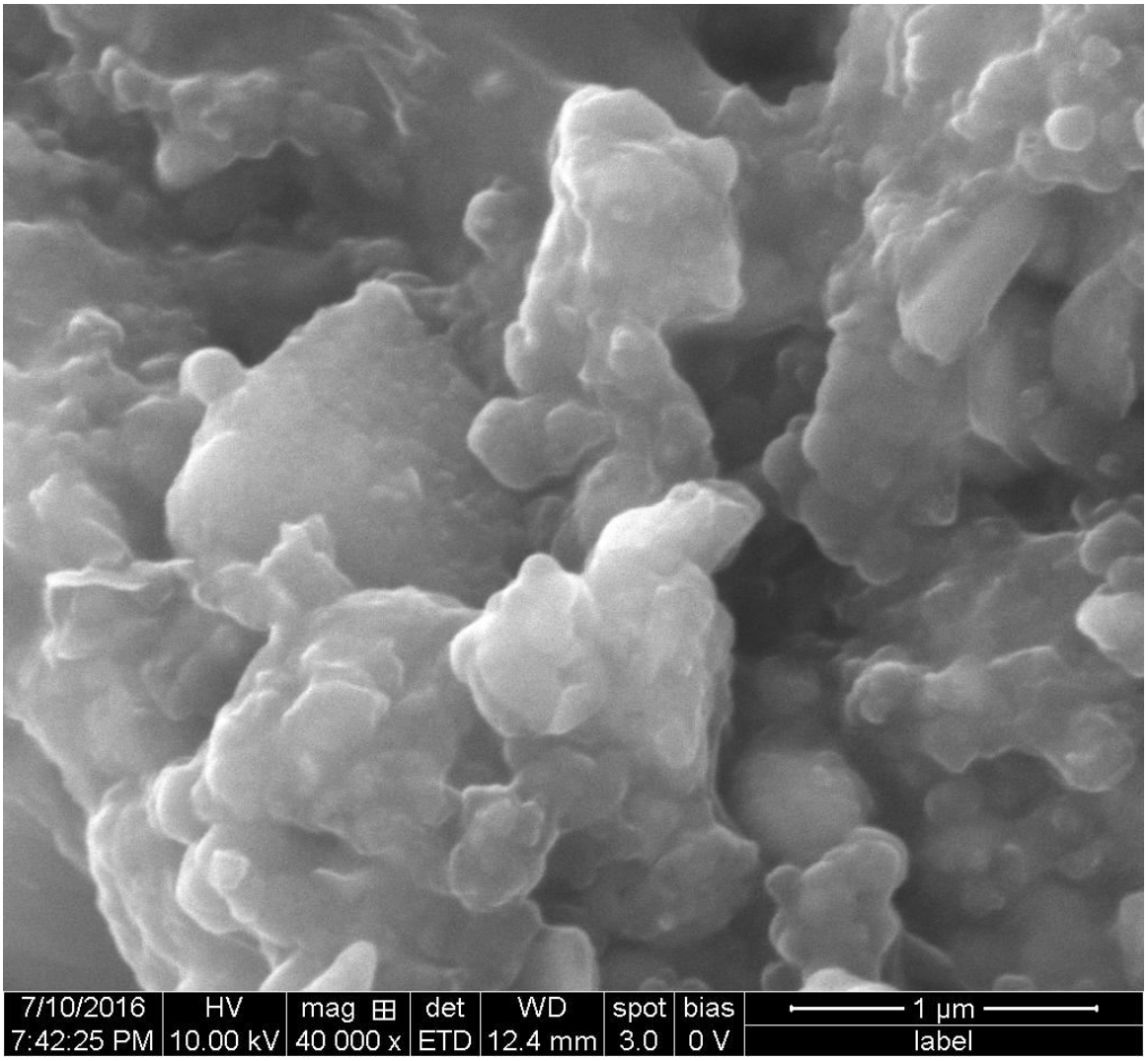


Fig no: 34. FESEM imagesof the prepared nanoemulsion.

7.7.4 In-vitro releae study

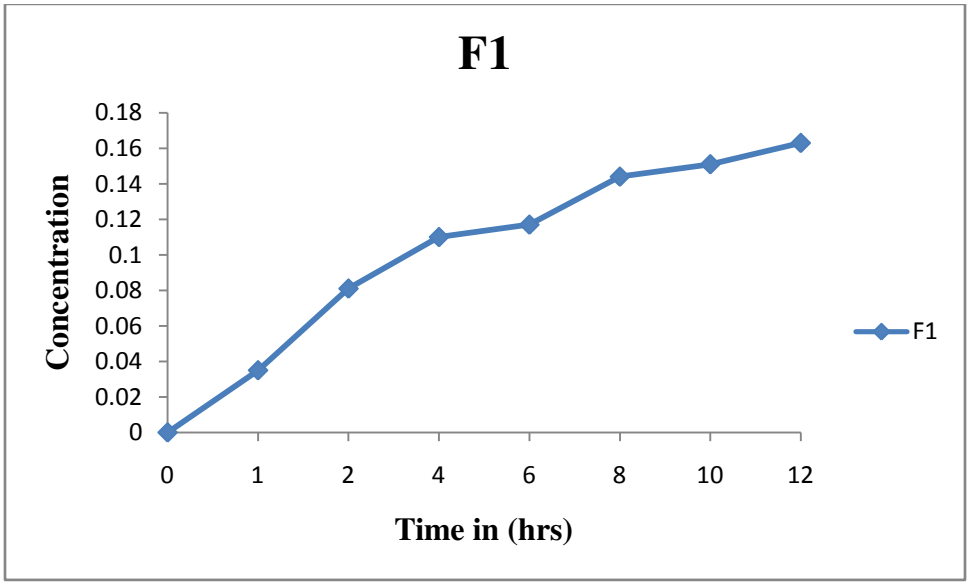
The release study was carried out as per the procedure described in materials and methods section 6.7.4.

The drug release was slow sustained and dependent upon the drug. Among the nine formulations F6 showed the maximum absorbance value during the 12 hr release study.

Table no: 14. Release profile of F1 batch.

Time in (hrs)	Concentration
0	0
1	0.035
2	0.081
4	0.110
6	0.117
8	0.144
10	0.151
12	0.163

Fig no: 35. Release profile of F1 batch.

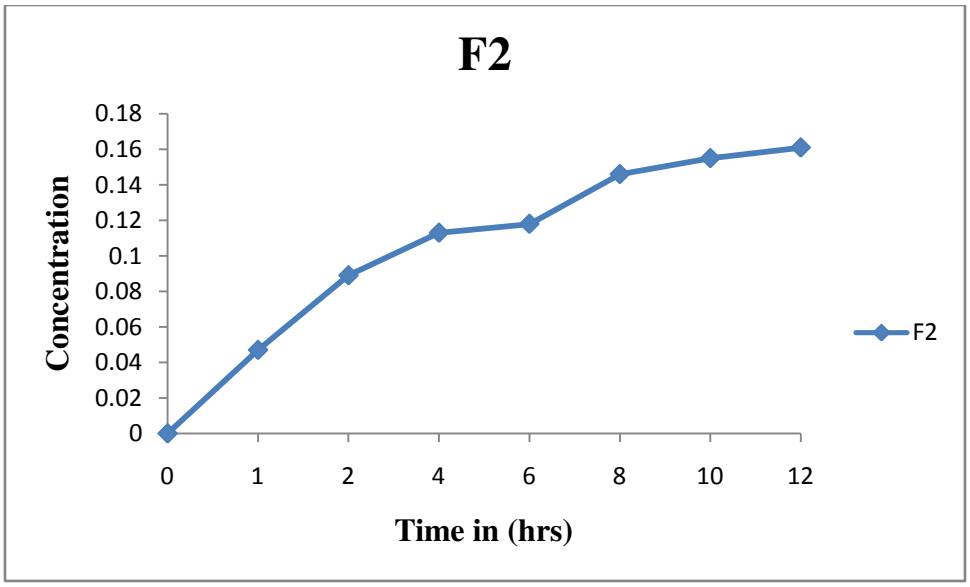


The drug release profile of F1batch was less when compared to F2 batch.

Table no: 15. Release profile of F2 batch.

Time in (hrs)	Concentration
0	0
1	0.047
2	0.089
4	0.113
6	0.118
8	0.146
10	0.155
12	0.161

Fig no: 36. Release profile of F3 batch

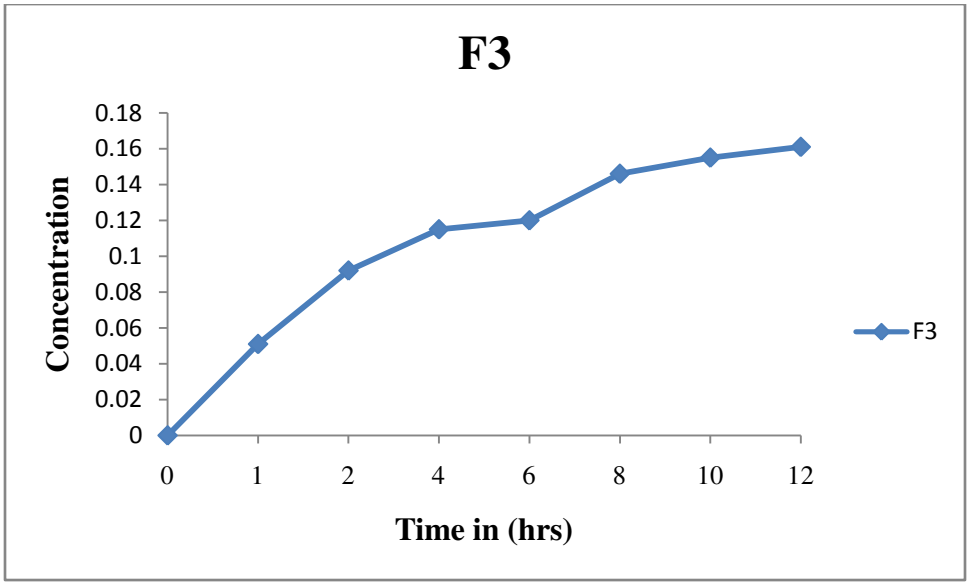


The drug release profile was better than F1 batch but comparatively less than F3 batch.

Table no: 16. Release profile of F3batch.

Time in (hrs)	Concentration
0	0
1	0.051
2	0.090
4	0.115
6	0.120
8	0.149
10	0.158
12	0.165

Fig no: 37. Release profile of F3 batch.

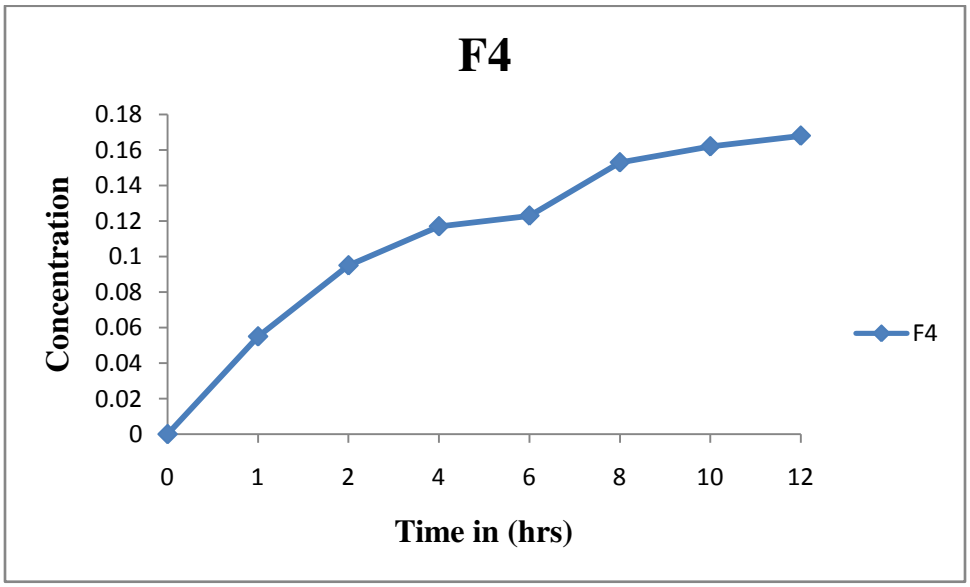


The drug release profile was less than F4 batch.

Table no: 17. Release profile of F4batch.

Time in (hrs)	Concentration
0	0
1	0.055
2	0.095
4	0.117
6	0.123
8	0.153
10	0.162
12	0.168

Fig no: 38. Release profile of F4 batch.

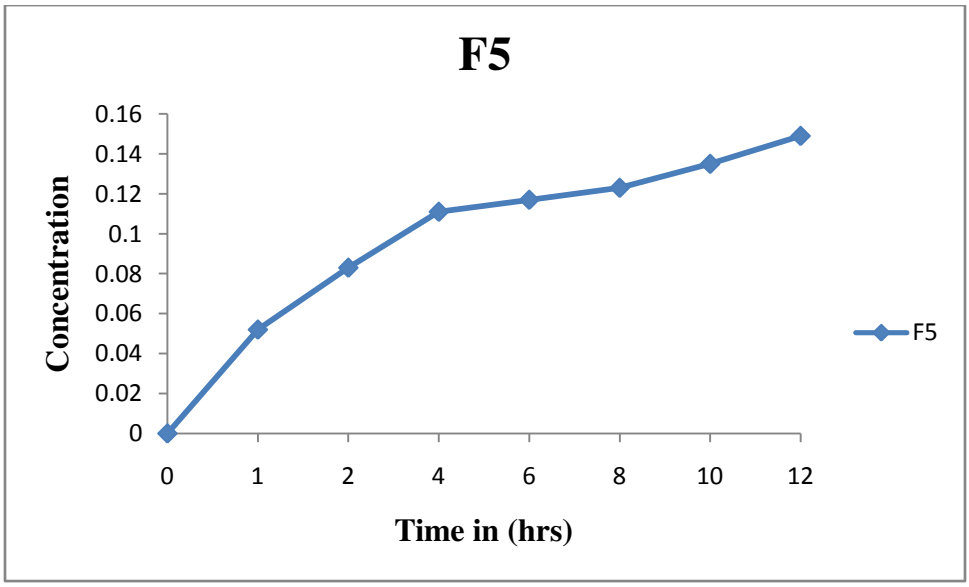


The drug release was uniform but less than F5 batch.

Table no: 18. Release profile of F5 Batch.

Time in (hrs)	Concentration
0	0
1	0.052
2	0.083
4	0.111
6	0.117
8	0.123
10	0.135
12	0.149

Fig no: 39. Release profile of F5 batch.

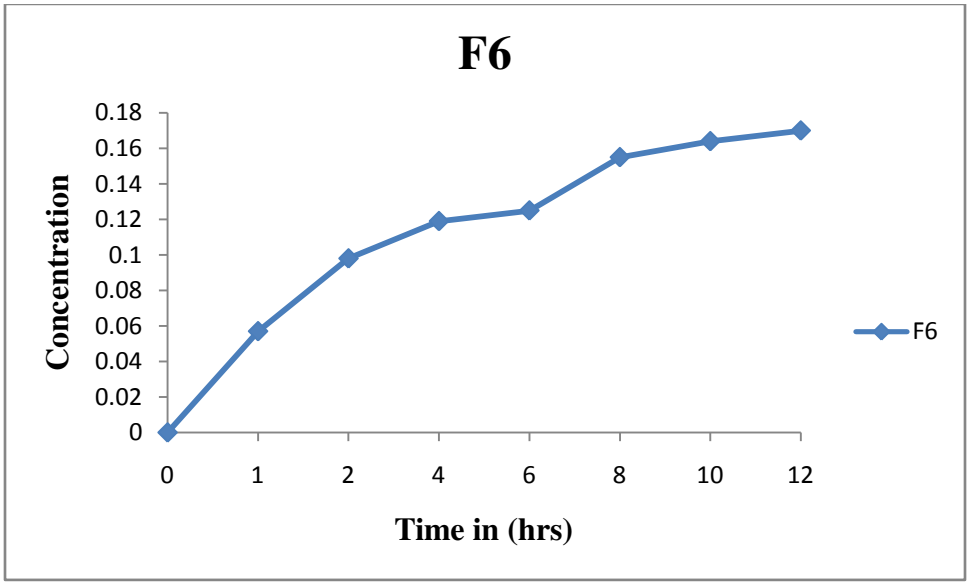


The drug release was uniform and good when compared with the earlier batches but less than F6 batch**.**

Table no: 19. Release profile of F6 batch.

Time in (hrs)	Concentration
0	0
1	0.057
2	0.098
4	0.119
6	0.125
8	0.155
10	0.164
12	0.170

Fig no: 40. Release profile of F6 batch.

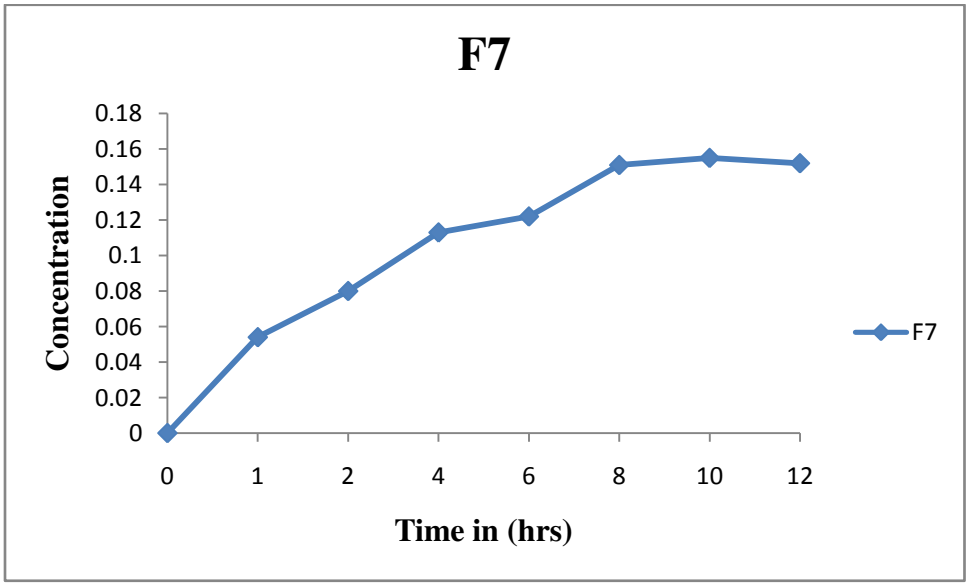


The release was uniform and good. This batch showed the highest release in the study.

Table no: 20. Release profile of F7 batch.

Time in (hrs)	Concentration
0	0
1	0.054
2	0.080
4	0.113
6	0.122
8	0.150
10	0.155
12	0.152

Fig no: 41. Release profile of F7 batch.

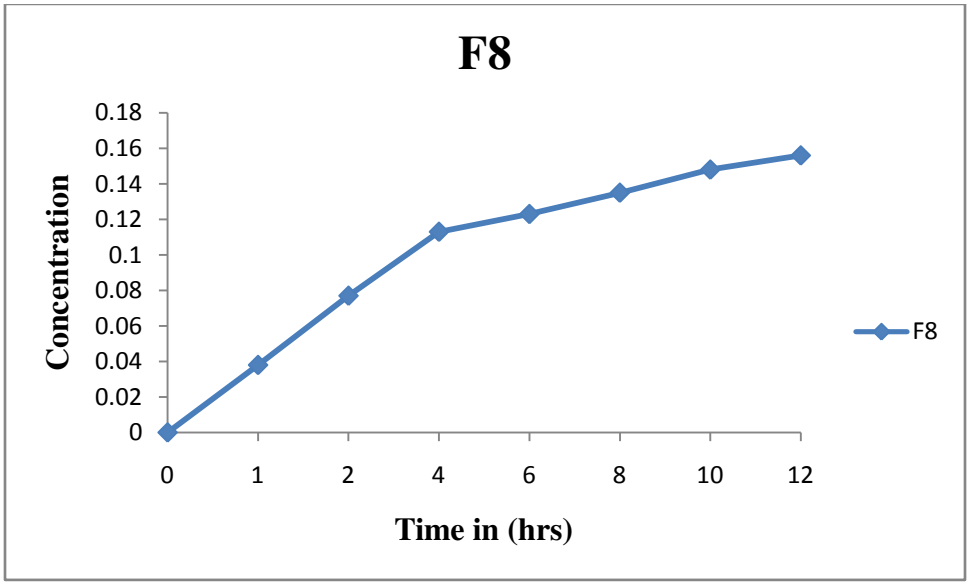


The drug release was less when compared to the F6 batch**.**

Table no: 21. Release profile of F8 batch.

Time in (hrs)	Concentration
0	0
1	0.038
2	0.017
4	0.113
6	0.123
8	0.135
10	0.148
12	0.156

Fig no: 42. Release profile of F8 batch.

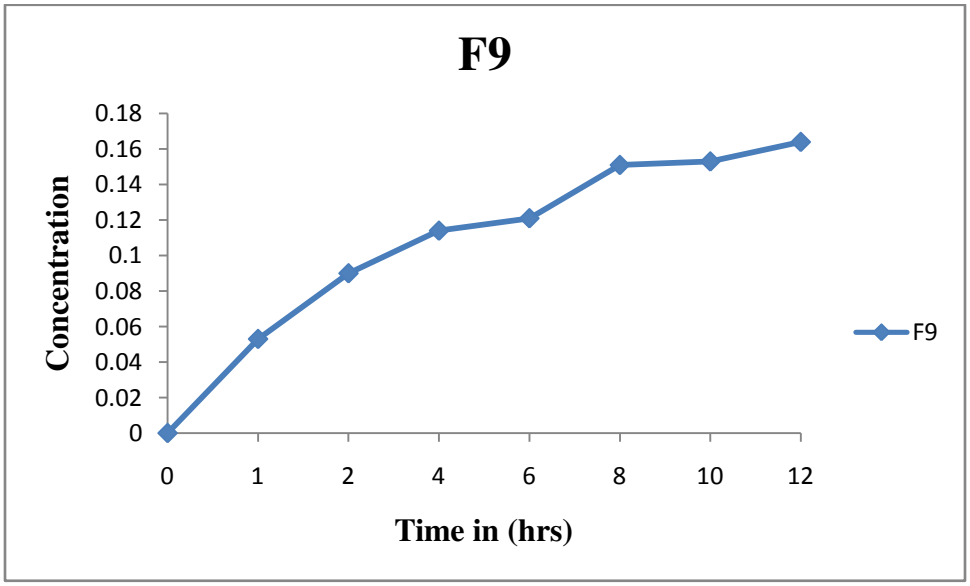


The drug release was uniform but less when compared to the F6 batch**.**

Table no: 22. Release profile of F9 batch.

Time in (hrs)	Concentration
0	0
1	0.053
2	0.090
4	0.114
6	0.121
8	0.151
10	0.153
12	0.164

Fig no: 43. Release profile of F9 batch.

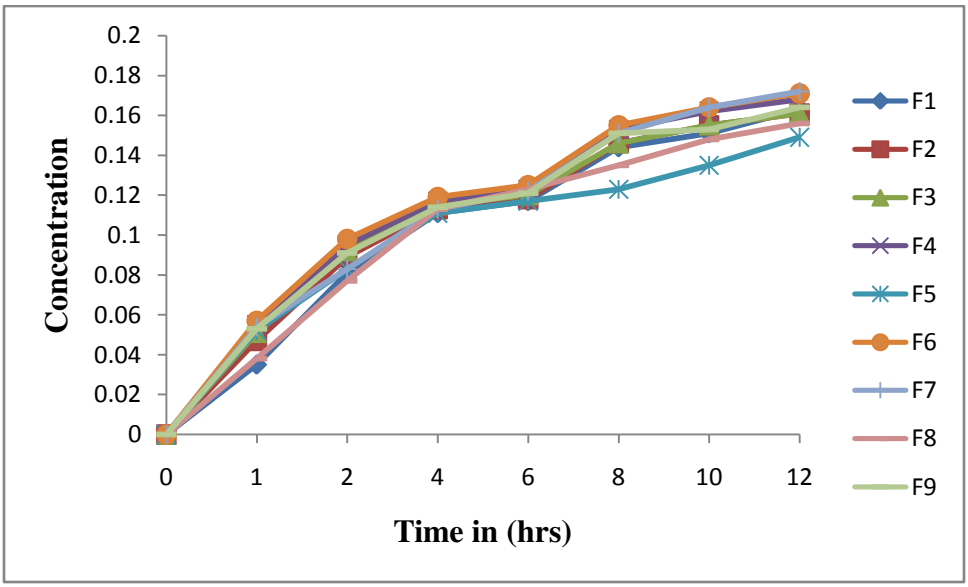


There was a decline in the release when compared to F6 batch. So, F6 is selected as the optimized batch.

Table no: 23. Cumulative in-vitro release profile of the prepared nanoemulsion.

Time in hours	Concentration								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
1	0.035	0.047	0.051	0.055	0.052	0.057	0.054	0.038	0.053
2	0.081	0.089	0.090	0.095	0.083	0.098	0.080	0.077	0.090
4	0.110	0.113	0.115	0.117	0.111	0.119	0.113	0.112	0.114
6	0.117	0.118	0.120	0.123	0.117	0.125	0.122	0.123	0.121
8	0.144	0.146	0.149	0.153	0.123	0.155	0.150	0.135	0.151
10	0.152	0.155	0.158	0.162	0.135	0.164	0.155	0.148	0.153
12	0.163	0.161	0.165	0.168	0.149	0.170	0.152	0.156	0.164

Fig no: 44. Cumulative in-vitro release profile of the prepared nanoemulsion.



7.8 Stability study of nanoemulsion

7.8.1 Mechanical stress study

The stability was determined by the procedure given under materials and method section 6.8.1.

The different nanoemulsion formulations were assessed for its stability by subjecting them at mechanical stress conditions (centrifugation at 2000 rpm). The effect of mechanical stress conditions on the physical stability of the nanoemulsion was observed by determining the percent phase separation, breaking of nanoemulsion or any physical change. The studies revealed that there is no change in the formulations even after 60 mins centrifugation at 2000 rpm.

Table no: 24. Stability study by mechanical stress method.

Centrifugation time (min)	% phase separated after centrifugation								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-

7.8.2 Accelerated temperature study

Accelerated temperature study was performed to assess the stability of formulations at different temperatures by storing the formulations at different temperatures by storing the formulations at 4 ± 1°C, 25±1°C and 40±1°C for 1, 10, 20 and 30 days and the degree of phase separation or any physical change was observed in the formulations. The studies revealed that there is no change in the physical state of the nanoemulsion on storing them at 4±1°C and 25±1°C till 30 days but breaking of the nanoemulsions was observed at 40±1°C after 10 days of storing.

Table no: 25. Stability study by accelerated temperature method.

Temp/days	% phase separation								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
4±1°C									
1	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-
25±1°C									
1	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
20	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0
30	1.0	1.0	1.0	1.5	1.5	1.5	2.0	2.0	2.0
40±1°C									
1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
10									
20	Breaking of nanoemulsion								
30									

SUMMARY AND CONCLUSION

The present study was a satisfactory attempt to formulate the nanoemulsion with a view of improving the oral bioavailability and thus giving a prolonged release of drug.

The main objective of the present study was to prepare an nanoemulsion of *Bauhinia racemosa* leaf extract for testing thrombolytic activities. No scientific study was made so far with nanoemulsion for the above activity.

The leave of *Bauhinia racemosa* were collected and identified . They were then extracted with ethanol as solvent for 8 hours using soxhlet apparatus by hot continous extraction method. The yield was found to be 15.95% w/v.

Oral acute toxicity was carried out as per OECD guidelines 423 by using rat as an animal model. A 2000 mg/kg body weight of animal dose is administered for acute toxicity study. In these studies the following parameters were observed for the evaluation of acute toxicity , which include gross behavior study, body weight analysis, hematological parameters, bio-chemical parameters, gross necropsy and histopathological studies. From the data of the acute toxicity studies it clearly demonstrates that the extract was safe up to 2000 mg/kg body weight of animal.

The thrombolytic activity was carried out by in-vitro method and gave appreciable results. The extract showed activity close to that of the standard.

Compatibility study was carried out by screening the components for solubility by visual observations.

The nanoemulsion was prepared in 9 batches with varying oil: water ratios like 2:1, 3:1, 4:1. The batch that showed a uniform and continuous high release was selected as the optimized batch. F6 was selected as the optimized batch.

The other evaluation parameters of nanoemulsion like paricle size, zeta potential, particle morphology study was done only for the optimized batch. The particle size and zeta potential reports were good.

The FESEM images showed good reports but the reports would have been still better with the use of Cryo SEM technique. But it is not available in Coimbatore and it takes time to get reports from other centers with Cryo SEM.

The stability study shows satisfactory results. There was no phase separation, breaking of nano emulsion or any physical changes in the nanoemulsion during the mechanical stress study. The nanoemulsion was stable at the temperature of 4±1°C, 25±1°C.Breaking of nanoemulsion was noted at 40±1°C.

Therefore this study was concluded that the *Bauhinia racemosa* extract loaded nanoemulsion drug delivery is effective for the treatment of thrombosis.

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